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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 1/00, 1/11, 1/19, 1/38, 15/11, 15/70, 15/74, 15/79, 15/81, 15/82, 15/85, C07H 21/02, 21/04 — A1

(11) International Publication Number: WO 00/32748

(43) International Publication Date: 8 June 2000 (08.06.00)

(21) International Application Number: PCT/US99/28592

(22) International Filing Date: 1 December 1999 (01.12.99)

(30) Priority Data:
60/110,616 2 December 1998 (02.12.98) US

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(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: GENE NETWORKS FOR CONTROL OF GENE EXPRESSION

(57) Abstract

The present invention relates to compositions for regulating gene expression in a cell. In particular, the invention provides composition constructs which switch expression of a gene of interest between stable "on" or "off" states in response to a transiently applied agent. The invention further provides adjustable–threshold switch constructs for expressing a gene of interest in response to the sustained application of an agent at a concentration above or below a desired threshold concentration. The invention also provides multi–state oscillator constructs in which expression of a gene of interest is periodically altered in the absence of administration of agents which are extraneous to the construct. Also provided are methods for using the invention's constructs.

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GENE NETWORKS FOR CONTROL OF GENE EXPRESSION

FIELD OF THE INVENTION

The present invention relates to compositions for regulating gene expression in a cell. In particular, the invention provides toggle switch constructs which switch expression of a gene of interest between stable "on" or "off" states in response to a transiently applied agent. The invention further provides adjustable-threshold switch constructs for expressing a gene of interest in response to the sustained application of an agent at a concentration above or below a desired threshold concentration. The invention also provides multi-state oscillator constructs in which expression of a gene of interest is periodically altered in the absence of administration of agents which are extraneous to the construct. Also provided are methods for using the invention's constructs.

BACKGROUND OF THE INVENTION

Cellular functions are dictated by the interaction of the products of multiple genes, including the repeated switching "on" and "off" of gene expression. While traditional genetic engineering approaches have been used to manipulate gene expression, these approaches are typically limited to the manipulation of single genes, and therefore do not recapitulate the multi-gene interactions which are responsible for cellular functions.

In particular, gene therapy approaches to treating disease are becoming increasingly practical in view of the development of improved gene delivery mechanisms [Anderson, Nature 392 (Suppl.), 25-30 (1998); Fan *et al.*, Nat. Biotechnol. 17, 870-872 (1999); Harding, *et al.*, Nat. Biotechnol. 16, 553--555 (1998); Kasahara *et al.*, Science 266, 1373-1376 (1994); Rendahl *et al.*, Nat. Biotechnol. 16, 757-761 (1998); Rivera *et al.*, Proc. Natl. Acad. Sci. USA 96, 8657-8662 (1999); Subramanian *et al.*, Nat. Biotechnol. 17, 873-877 (1999)], the construction of novel gene-expression systems [Baron *et al.*, Nucleic Acids Res. 23, 3605-3606 (1995); Gossen & Bujard, Proc. Natl. Acad. Sci. USA 89, 5547-5551 (1992); Gossen *et al.*, Science 268, 1766--1769 (1995); Rivera *et al.*, Nat. Med. 2, 1028-1032], the intensifying pace of human genome sequencing [Marshall, Science 284, 1439-1441 (1999); Strohman, Nat. Biotechnol. 17, 112 (1999); Wadman M., Nature 398, 177 (1999)], and the development of techniques for the rapid identification of protein function [Nordhoff *et al.*, Nat. Biotechnol. 17, 884-888 (1999); Norman *et al.*, Science 285,

591-595 (1999); Geyer *et al.*, Proc. Natl. Acad. Sci. USA 96, 8567-8572 (1999)]. However, the range of regulatory functions permitted by current gene-expression systems is limited to the up- or down-regulation of gene expression in response to a sustained small-molecule signal. This limitation restricts the reach of gene therapy to the replacement of defective single genes and also precludes the repair or augmentation of complete gene regulatory "circuits."

An additional drawback of current expression systems is their reliance upon the development of regulatory proteins with novel functionality [Gossen *et al.* (1992) *supra*; Gossen *et al.* (1995) *supra*; Rivera *et al.* (1999) *supra*]. Thus, the construction and tuning of these regulatory systems is hindered by the bottleneck of protein engineering which is a time-consuming and uncertain process. This type of engineering also does not include the use of synthetic gene networks to produce regulatory behavior that is qualitatively distinct from its component elements.

Thus, what is needed are methods and compositions for regulating gene expression and in particular for investigating the interaction of multiple genes.

SUMMARY OF THE INVENTION

The invention provides methods and compositions for regulating gene expression. The invention provides a method of altering transcription of a gene of interest, comprising: (a) providing: (i) a host cell; (ii) a composition comprising a first construct and a second construct, wherein: (a) the first construct comprises a first constitutive promoter operably linked to a first gene encoding a first protein and to a first gene of interest; and (b) the second construct comprises a second constitutive promoter operably linked to a second gene encoding a second protein, wherein the first protein represses transcription from the second promoter and the second protein represses transcription from the first promoter; (iii) a first agent capable of reducing repression of transcription from the first promoter; and (iv) a second agent capable of reducing repression of transcription from the second promoter; (b) transfecting the host cell with the composition; (c) exposing the transfected cell in any order to: (i) the first agent such that transcription of the first gene of interest is induced compared to transcription in the absence of the first agent; and (ii) the second agent such that transcription of the first gene of interest is repressed compared to transcription in the absence of the second agent. Without limiting the invention to any particular mode of exposing, in one embodiment, the exposing is transient. Without intending to limit the

invention to a particular host cell, in an alternative embodiment, the host cell is prokaryotic. In a more preferred embodiment, the prokaryotic cell is *Escherichia coli*. In another preferred embodiment, the host cell is eukaryotic. In a more preferred embodiment, the eukaryotic host cell is selected from the group consisting of yeast and human. In yet another embodiment, the second construct further comprises a second gene of interest operably linked to said second constitutive promoter, the exposing of the transfected cell to the first agent results in repressed transcription of the second gene of interest compared to transcription in the absence of the first agent, and the exposing of the transfected cell to the second agent results in induced transcription of the second gene of interest compared to transcription in the absence of the second agent.

The invention also provides a method of increasing transcription of a gene of interest, comprising: (a) providing: (i) a host cell; (ii) a composition comprising a first construct and a second construct, wherein: (a) the first construct comprises an inducible promoter operably linked to a first gene encoding a first protein and to a gene of interest; and (b) the second construct comprises a constitutive promoter operably linked to a second gene encoding a second protein, wherein the second protein represses transcription from the first promoter and the first protein represses transcription from the second promoter; (iii) an agent capable of activating transcription from the first promoter in the composition; (b) transfecting the host cell with the composition; and (c) exposing the host cell to a concentration equal to or greater than a a threshold concentration of the agent such that transcription of the gene of interest is increased compared to transcription in the presence of the agent at a concentration greater than the threshold concentration. While not intending to limit the invention to a particular host cell, in one embodiment, the host cell is prokaryotic. In a preferred embodiment, the prokaryotic host cell is *Escherichia coli*. In an alternative embodiment, the host cell is eukaryotic. In a more preferred embodiment, the eukaryotic host cell is selected from the group consisting of yeast and human.

Also provided by the invention is a method of increasing transcription of a gene of interest, comprising: (a) providing: (i) a host cell; (ii) a composition comprising a first construct and a second construct, wherein: (a) the first construct comprises an inducible promoter operably linked to a first gene encoding a first protein; and (b) the second construct comprises a constitutive promoter operably linked to a gene of interest and to a second gene encoding a second protein, wherein the second protein represses transcription from the first promoter and the first protein represses transcription from the second

promoter; (iii) an agent capable of activating transcription from the first promoter in the composition; (b) transfecting the host cell with the composition; and (c) exposing the host cell to at least a threshold concentration of the agent such that transcription of the gene of interest is increased compared to transcription in the presence of the agent at a concentration below the threshold concentration. While it is not intended that the invention be limited to any particular host cell, in one embodiment, the host cell is prokaryotic. In a preferred embodiment, the prokaryotic host cell is *Escherichia coli*. In an alternative embodiment, the host cell is eukaryotic. In a preferred embodiment, the eukaryotic host cell is selected from the group consisting of yeast and human.

The invention further provides a method of expressing a gene of interest, comprising: (a) providing: (i) a host cell; (ii) a composition comprising a first construct, second construct and third construct, wherein: (a) the first construct comprises a first inducible promoter operably linked to a first gene encoding a first protein and to a gene of interest; and (b) the second construct comprises a constitutive promoter operably linked to a second gene encoding a second protein and to a third gene encoding a third protein; (c) the third construct comprises a second inducible promoter operably linked to a fourth gene encoding a fourth protein, wherein: (i) the first protein represses transcription from the constitutive promoter; (ii) the second protein represses transcription from the first inducible promoter; (iii) the third protein increases transcription from the third promoter; and (iv) the fourth protein increases transcription from the first inducible promoter; (b) transfecting the host cell with the composition; and (c) culturing the transfected cell such that the gene of interest is expressed. While not limiting the invention to any particular type of expression, in one embodiment, the expression is periodic. It is not intended that the invention be limited to a particular type of host cell. However, in one embodiment, the host cell is prokaryotic. In an alternative embodiment, the host cell is eukaryotic.

Also provided herein is a method of expressing a gene of interest, comprising: (a) providing: (i) a host cell; (ii) a composition comprising a first construct, second construct and third construct, wherein: (a) the first construct comprises an inducible promoter operably linked to a first gene encoding a first protein, a second gene encoding a second protein, and to a gene of interest; and (b) the second construct comprises a constitutive promoter operably linked to a third gene encoding a third protein; and (c) the third construct comprises a third promoter operably linked to a fourth gene encoding a fourth protein, wherein: (i) the first protein represses transcription from the constitutive promoter; (ii) the

second protein represses transcription from the third promoter; (iii) the third protein represses transcription from the inducible promoter; and (iv) the fourth protein increases transcription from the inducible promoter; (b) transfecting the host cell with the composition; and (c) culturing the transfected cell such that the gene of interest is expressed. Without intending to limit the invention to a particular type of expression, in one embodiment, the expression is periodic. While not limiting the invention to a particular type of host cell, in one preferred embodiment, the host cell is prokaryotic. In another preferred embodiment, the host cell is eukaryotic.

The invention additionally provides a method of expressing a gene of interest, comprising: (a) providing: (i) a host cell; (ii) a composition comprising a first construct, second construct and third construct, wherein: (a) the first construct comprises an inducible promoter operably linked to a first gene encoding a first protein and to a gene of interest; (b) the second construct comprises a first constitutive promoter operably linked to a second gene encoding a second protein; and (c) the third construct comprises a second constitutive promoter operably linked to a third gene encoding a third protein, wherein: (i) the first protein represses transcription from the first and second constitutive promoters; (ii) the second protein represses transcription from the inducible promoter; and (iii) the third protein increases transcription from the inducible promoter; (b) transfecting the host cell with the composition; and (c) culturing the transfected cell such that the gene of interest is expressed.

In addition to the above-described methods, the invention further provides compositions for regulating gene expression. The invention provides a composition comprising a first construct and a second construct, wherein: (a) the first construct comprises a first promoter operably linked to a first gene and to a first gene of interest, wherein the first gene encodes a first protein and the first gene of interest encodes a first protein of interest; and (b) the second construct comprises a second promoter operably linked to a second gene wherein the second gene encodes a second protein, wherein: (i) the first and second promoters are constitutive; (ii) the first protein is capable of multimerizing to form a first protein homo-dimer, the first protein homo-dimer being capable of binding to the second promoter, wherein the binding represses transcription of the second gene and of the gene of interest; and (iii) the second protein is capable of multimerizing to form a second protein homo-dimer, the second protein homo-dimer being capable of binding to the first promoter, wherein the binding represses transcription of the first gene and of the gene of interest. In one embodiment, based on the inventors' preliminary analysis, the strength of

each of the first promoter (α_1) and of the second promoter (α_2) has a value greater than two. In another embodiment, the α_1 and α_2 are balanced. In yet another embodiment, the second construct further comprises a second gene of interest operably linked to the second promoter and wherein the second gene of interest encodes a second protein of interest.

In yet a further embodiment, at least one of the first and second promoters is derived from a prokaryotic cell. In a preferred embodiment, the prokaryotic cell is *Escherichia coli*. In a more preferred embodiment, at least one of the first and second promoters is derived from a nucleic acid sequence selected from the group consisting of P_L promoter, P_{trc} promoter, arsenic operon, ASC operon, sucrose operon, deoxyribose operon, DGORKAT operon, fructose operon, galactose operon, galactitol operon, SOS response regulon, raffinose operon, and tetracycline resistance operon.

In another embodiment, at least one of the first and second promoters is derived from a virus. In a preferred embodiment, the virus is selected from the group consisting of human cytomegaolvirus, herpes simplex virus, and simian virus 40. In a more preferred embodiment, at least the one of the first and second promoters is selected from the group consisting of P_{hCMV}, P_{HSVtk}, and P_{SV40}.

In yet another embodiment, at least one of the first and second promoter is derived from a eukaryotic cell. In a more preferred embodiment, the eukaryotic cell is selected from the group consisting of human cell and yeast cell.

In yet a further preferred embodiment, at least the constitutive second promoter is operably linked to a first operator sequence and the inducible first promoter is operably linked to a second operator sequence, and wherein the constitutive second promoter and the inducible first promoter is derived from a first organism, and the first and second operator sequences are derived from a second organism. In a preferred embodiment, at least one of the first promoter and the second promoter is derived from a first organism, and the operator sequence is derived from a second organism. In an alternative preferred embodiment, the first and second organisms are the same. In a more preferred embodiment, the first and second organisms are prokaryotic. In an alternative more preferred embodiment, the first and second organisms are eukaryotic. In a further preferred embodiment, the first and second prokaryotic organisms are *Escherichia coli*. In another preferred embodiment the first and second organisms are different. In a more preferred embodiment, at least one of the first and second organisms is a prokaryotic organism. In a further preferred embodiment, at least one of the first and second organisms is a eukaryotic organism. In yet a further preferred

embodiment, the prokaryotic organism is *Escherichia coli*. In another preferred embodiment, the first organism is a virus, and the second organism is a prokaryotic organism. In a more preferred embodiment, the prokaryotic organism is *Escherichia coli*. In yet a more preferred embodiment, the operator sequence is selected from the group consisting of O_{lac}, O_{lex}, O_{tet1}, O_{tet2}, O_{R1}, O_{R2}, O_{R3}, O_{L1}, O_{L2} and O_{L3}. In another more preferred embodiment, the virus is selected from the group consisting of human cytomegalovirus, herpes simplex virus and simian virus 40. In yet a more preferred embodiment, the viral promoter is selected from the group consisting of P_{hCMV}, P_{HSVtk}, and P_{SV40}.

The invention also provides a composition comprising a first construct and a second construct, wherein: (a) the first construct comprises a first promoter operably linked to a first gene and to a first gene of interest, wherein the first gene encodes a first protein and the first gene of interest encodes a first protein of interest; and (b) the second construct comprises a second promoter operably linked to a second gene wherein the second gene encodes a second protein, wherein: (i) the first and second promoters are constitutive; (ii) the first protein is capable of multimerizing to form a first protein homo-multimer, wherein the first protein homo-multimer is other than homo-dimer and is capable of binding to the second promoter, wherein the binding represses transcription of the second gene; and (iii) the second protein is capable of multimerizing to form a second protein homo-multimer, wherein the second protein homo-multimer is other than homo-dimer and is capable of binding to the first promoter, wherein the binding represses transcription of the first gene and of the gene of interest.

In one embodiment, based on the inventors' preliminary determination, the strength of each of the first promoter (α_1) and of the second promoter (α_2) has a value greater than one. In a preferred embodiment, the $\alpha 1$ and $\alpha 2$ are balanced. In another embodiment, the second construct further comprises a second gene of interest operably linked to the second promoter and wherein the second gene of interest encodes a second protein of interest. In yet another embodiment, at least one of the first and second promoters is derived from a prokaryotic organism. In a preferred embodiment, the prokaryotic organism is *Escherichia coli*. In a more preferred embodiment, at least one of the first and second promoters is derived from a nucleic acid sequence selected from the group consisting of P_L promoter, P_{trc} promoter, arsenic operon, ASC operon, sucrose operon, deoxyribose operon, DGORKAT

operon, fructose operon, galactose operon, galactitol operon, SOS response regulon, raffinose operon, and tetracycline resistance operon.

In another embodiment, at least one of the first and second promoters is derived from a virus. In a preferred embodiment, the virus is selected from the group consisting of human cytomegaolvirus, herpes simplex virus, and simian virus 40. In a more preferred embodiment, at least the one of the first and second promoters is selected from the group consisting of P_{hCMV}, P_{HSVtk}, and P_{SV40}.

In yet another embodiment, at least one of the first and second promoter is derived from a eukaryotic organism. In a preferred embodiment, the eukaryotic organism is selected from the group consisting of human and yeast. In yet a further embodiment, at least one of the first construct and the second construct further incorporates an operator sequence. In a preferred embodiment, the at least one of the first promoter and the second promoter is derived from a first organism, and the operator sequence is derived from a second organism. In an alternative preferred embodiment, the first and second organisms are the same. In a more preferred embodiment, the first and second organisms are prokaryotic organisms. In yet a more preferred embodiment, the first and second prokaryotic organisms are *Escherichia coli*. In another preferred embodiment, the first and second organisms are eukaryotic organisms. In yet another alternative preferred embodiment, the first and second organisms are different. In a more preferred embodiment, at least one of the first and second organisms is a prokaryotic organism. In yet a more preferred embodiment, the prokaryotic organism is *Escherichia coli*. In a further more preferred embodiment, at least one of the first and second organisms is a eukaryotic organism. In another alternative embodiment, the first organism is a virus, and the second organism is a prokaryotic organism. In a preferred embodiment, the prokaryotic organism is *Escherichia coli*. In a more preferred embodiment, the operator sequence is selected from the group consisting of O_{lac}, O_{lex}, O_{tet1}, O_{tet2}, O_{R1}, O_{R2}, O_{R3}, O_{L1}, O_{L2} and O_{L3}. In another preferred embodiment, the virus is selected from the group consisting of human cytomegalovirus, herpes simplex virus and Simian virus 40. In a further preferred embodiment, the viral promoter is selected from the group consisting of P_{hCMV}, P_{HSVtk}, and P_{SV40}.

Also provided by the invention is a composition comprising a first construct and a second construct, wherein: (a) the first construct comprises a first promoter operably linked to a first gene and to a first gene of interest, wherein the first promoter is inducible, the first gene encodes a first protein, and the first gene of interest encodes a first protein of interest;

and (b) the second construct comprises a second promoter operably linked to a second gene, wherein the second promoter is constitutive and the second gene encodes a second protein, wherein: (i) the first promoter is capable of binding to: (1) the second protein wherein the binding represses transcription of the first gene and of the gene of interest; and (2) an agent wherein the binding increases transcription of the first gene and of the gene of interest and increases the strength of the first promoter; and (ii) the second promoter is capable of binding to the first protein, wherein the binding represses transcription of the second gene.

In one embodiment, the second construct further comprises a second gene of interest operably linked to the second promoter and wherein the second gene of interest encodes a second protein of interest. In another embodiment, (i) the first protein is capable of multimerizing to form a first protein homo-dimer; (ii) the second protein is capable of multimerizing to form a second protein homo-dimer. Optionally, based on the inventors' preliminary determination, the maximum strength of the first promoter (α_1) and the strength of the second promoter (α_2) has a value greater than two.

In another embodiment, (i) the first protein is capable of multimerizing to form a first protein homo-multimer other than homo-dimer; and (ii) the second protein is capable of multimerizing to form a second protein homo-multimer other than homo-dimer. Optionally, based on the inventors' preliminary determination, the maximum strength of the first promoter (α_1) and the strength of the second promoter (α_2) has a value greater than one. In yet another embodiment, at least one of the first and second promoters is derived from a eukaryotic cell. In a yet alternative embodiment, at least one of the first and second promoters is derived from a prokaryotic organism. In a preferred embodiment, the prokaryotic organism is *Escherichia coli*. In an alternative more preferred embodiment, the constitutive second promoter is derived from a nucleic acid sequence selected from the group consisting of P_L promoter, P_{trc} promoter, arsenic operon, ASC operon, sucrose operon, deoxyribose operon, DGORKAT operon, fructose operon, galactose operon, galactitol operon, SOS response regulon, raffinose operon, and tetracycline resistance operon.

In another preferred embodiment, at least one of the first and second promoters is derived from a virus. In a more preferred embodiment, the virus is selected from the group consisting of human cytomegaolvirus, herpes simplex virus, and simian virus 40. In a yet more preferred embodiment, the constitutive second promoter is selected from the group consisting of P_{hCMV}, P_{HSVtk}, and P_{SV40}.

In yet another preferred embodiment, at least one of the first and second promoter is derived from a eukaryotic organism. In a more preferred embodiment, the eukaryotic organism is selected from the group consisting of human and yeast.

In yet a further preferred embodiment, at least the constitutive second promoter is operably linked to a first operator sequence and the inducible first promoter is operably linked to a second operator sequence, and wherein the constitutive second promoter and the inducible first promoter is derived from a first organism, and the first and second operator sequences are derived from a second organism. In a more preferred embodiment, the first and second organisms are the same. In a yet more preferred embodiment, the first and second organisms are prokaryotic organisms. In a yet even more preferred embodiment, the first and second prokaryotic organisms are *Escherichia coli*. In an alternative even more preferred embodiment, the first and second organisms are eukaryotic organisms. In another more preferred embodiment, the first and second organisms are different. In a yet more preferred embodiment, at least one of the first and second organisms is a prokaryotic organism.In a yet even more preferred embodiment, the prokaryotic organism is *Escherichia coli*. In another yet more preferred embodiment, at least one of the first and second organisms is a eukaryotic organism. In an alternative yet another more preferred embodiment, the first organism is a virus, and the second organism is a prokaryotic organism. In a yet more preferred embodiment, the prokaryotic organism is *Escherichia coli*. In a yet even more preferred embodiment, the operator sequence is selected from the group consisting of O_{lac}, O_{lex}, O_{tet1}, O_{tet2}, O_{R1}, O_{R2}, O_{R3}, O_{L1}, O_{L2} and O_{L3}. In a yet even more preferred embodiment, the constitutive second promoter is derived from a nucleic acid sequence selected from the group consisting of P_L promoter, P_{trc} promoter, arsenic operon, ASC operon, sucrose operon, deoxyribose operon, DGORKAT operon, fructose operon, galactose operon, galactitol operon, SOS response regulon, raffinose operon, and tetracycline resistance operon. In an alternative even more preferred embodiment, the inducible first promoter is derived from a nucleic acid sequence selected from the group consisting of arabanose operon, CAD operon, deoP2, Cyn operon, Dsd operon, formate dehydrogenase/hydrogenase operon, malPp, monoamine oxidase operon, IlvC operon, and urease operon. In another yet even more preferred embodiment, the virus is selected from the group consisting of human cytomegalovirus, herpes simplex virus and simian virus 40. In a further preferred embodiment, the promoter is selected from the group consisting of P_{hCMV}, P_{HSVtk}, and P_{SV40}.

In yet another preferred embodiment, the agent is a fusion protein comprising an activation domain and a DNA binding domain. In a mo re preferred embodiment, the activation domain is derived from an organism selected from the group consisting of virus and eukaryotic organism. In a yet more preferred embodiment, the virus is a herpes simplex virus. In a yet even more preferred embodiment, the activation domain is VP16. In another yet even more preferred embodiment, the eukaryotic organism is human. In a more preferred embodiment, the activation domain is B42. In a yet mor preferred embodiment, the eukaryotic organism is yeast. In a pareferred embodiment, the activation domain is GAL4. In another more preferred embodiment, the DNA binding domain is derived from a protein wherein the protein is derived from a prokaryotic organism. In yet a more preferred embodiment, the prokaryotic organism is *Escherichia coli*. In a yet even more preferred embodiment, the protein is selected from the group consisting of ArsR, AscG, CscR, DeoR, DgoR, FruR, GalR, GatR, LexA, CI, LacI, RafR, and TetR.

The invention also provides a composition comprising a first construct and a second construct, wherein: (a) the first construct comprises a first promoter operably linked to a first gene, wherein the first promoter is inducible, and the first gene encodes a first protein; and (b) the second construct comprises a second promoter operably linked to a second gene and to a first gene of interst, wherein the second promoter is constitutive, the second gene encodes a second protein, and the first gene of interst encodes a first protein of interest, wherein: (i) the first promoter is capable of binding to: (1) the second protein wherein the binding represses transcription of the first gene; and (2) an agent wherein the binding increases transcription of the first gene and increases the strength of the first promoter; and (ii) the second promoter is capable of binding to the first protein, wherein the binding represses transcription of the second gene and of the first gene of interst. In one embodiment, the first construct further comprises a second gene of interest operably linked to the first promoter and wherein the second gene of interest encodes a second protein of interest.

The invention additionally provides a composition comprising a first construct, second construct and third construct, wherein: (a) the first construct comprises a first promoter operably linked to a first gene and to a gene of interest, wherein the first promoter is inducible, the first gene encodes a first protein, and the gene of interest encodes a protein of interest; and (b) the second construct comprises a second promoter operably linked to a second gene and to a third gene wherein the second promoter is constitutive, the second

gene encodes a second protein and the third gene encodes a third protein; (c) the third construct comprises a third promoter operably linked to a fourth gene, wherein the third promoter is inducible and wherein the fourth gene encodes a fourth protein, wherein: (i) the first promoter is capable of binding to: (1) the second protein wherein the binding represses transcription of the first gene and of the first gene of interest; and (2) the fourth protein wherein the binding increases transcription of the first gene and of the first gene of interest and increases the strength of the first promoter; (ii) the second promoter is capable of binding to the first protein and wherein the binding represses transcription of the second gene; and (iii) the third promoter is capable of binding to the third protein wherein the binding increases transcription of the fourth gene.

In one embodiment, the second construct further comprises a second gene of interest operably linked to the second promoter and wherein the second gene of interest encodes a second protein of interest. In another embodiment, at least one of the first and second promoters is derived from a prokaryotic organism. In a preferred embodiment, the prokaryotic organism is *Escherichia coli*. In a more preferred embodiment, the constitutive second promoter is derived from a nucleic acid sequence selected from the group consisting of P_L promoter, P_{trc} promoter, arsenic operon, ASC operon, sucrose operon, deoxyribose operon, DGORKAT operon, fructose operon, galactose operon, galactitol operon, SOS response regulon, raffinose operon, and tetracycline resistance operon.

In another embodiment, at least one of the first and second promoters is derived from a virus. In a preferred embodiment, the virus is selected from the group consisting of human cytomegaolvirus, herpes simplex virus, and simian virus 40. In a more preferred embodiment, the constitutive second promoter is selected from the group consisting of P_{hCMV}, P_{HSVtk}, and P_{SV40}.

In yet another embodiment, at least one of the first and second promoter is derived from a eukaryotic organism. In a preferred embodiment, the eukaryotic organism is selected from the group consisting of human and yeast.

In yet another embodiment, the fourth gene is derived from a prokaryotic organism. In a preferred embodiment, the prokaryotic organism is *Escherichia coli*. In a more preferred embodiment, the fourth gene encodes a protein selected from the group consisting of AraC, CadC, CRP, CynR, DsdC, FhlA, MalT, MaoB, IlvY, and UreR.

In yet another embodiment, the third gene is derived from a prokaryotic organism. In a preferred embodiment, the prokaryotic organism is *Escherichia coli.* In a more preferred embodiment, the third gene encodes a protein selected from the group consisting of AraC, CadC, CRP, CynR, DsdC, FhlA, MalT, MaoB, IlvY, and UreR.

In still another embodiment, the third promoter is derived from a virus. In a preferred embodiment, the virus is selected from the group consisting of human cytomegalovirus, herpes simplex virus and simian virus 40. In a more preferred embodiment, the third promoter is selected from the group consisting of P_{hCMV}, P_{HSVtk}, and P_{SV40}.

In yet another embodiment, the third promoter is derived from a eukaryotic organism.

In still another embodiment, the third promoter is derived from a prokaryotic organism. In a preferred embodiment, the prokaryotic organism is *Escherichia coli.* In a more preferred embodiment, the third promoter is derived from a nucleic acid sequence selected from the group consisting of arabanose operon, CAD operon, deoP2, Cyn operon, Dsd operon, formate dehydrogenase/hydrogenase operon, malPp, monoamine oxidase operon, IlvC operon, and urease operon.

In yet another embodiment, at least the constitutive second promoter is operably linked to a first operator sequence and the inducible first promoter is operably linked to a second operator sequence, and wherein the constitutive second promoter and the inducible first promoter is derived from a first organism, and the first and second operator sequences are derived from a second organism. In a preferred embodiment, the first and second organisms are the same. In a more preferred embodiment, the first and second organisms are prokaryotic organisms. In yet a more preferred embodiment, the first and second prokaryotic organisms are *Escherichia coli.* In an alternative yet more preferred embodiment, the first and second organisms are eukaryotic organisms. In an alternative preferred embodiment, the first and second organisms are different. In a more preferred embodiment, at least one of the first and second organisms is a prokaryotic organism. In a yet more preferred embodiment, the prokaryotic organism is *Escherichia coli.* In an alternative more preferred embodiment, at least one of the first and second organisms is a eukaryotic organism. In another alternative preferred embodiment, the first organism is a virus, and the second organism is a prokaryotic organism. In a more preferred embodiment, the prokaryotic organism is *Escherichia coli.* In an alterative yet more preferred embodiment, the constitutive second promoter is derived from a nucleic acid sequence

selected from the group consisting of P_L promoter, P_{trc} promoter, arsenic operon, ASC operon, sucrose operon, deoxyribose operon, DGORKAT operon, fructose operon, galactose operon, galactitol operon, SOS response regulon, raffinose operon, and tetracycline resistance operon. In yet another alternative more preferred embodiment, at least one of the inducible first promoter and the inducible third promoter is derived from a nucleic acid sequence selected from the group consisting of arabanose operon, CAD operon, deoP2, Cyn operon, Dsd operon, formate dehydrogenase/hydrogenase operon, malPp, monoamine oxidase operon, IlvC operon, and urease operon. In a yet more preferred embodiment, the operator sequence is selected from the group consisting of O_{lac}, O_{lex}, O_{tet1}, O_{tet2}, O_{R1}, O_{R2}, O_{R3}, O_{L1}, O_{L2} and O_{L3}. In an alternative more preferred embodiment, the virus is selected from the group consisting of human cytomegalovirus, herpes simplex virus and simian virus 40. In a yet more preferred embodiment, the viral promoter is selected from the group consisting of P_{hCMV}, P_{HSVtk}, and P_{SV40}.

In yet another embodiment, the third protein or the fourth protein is a fusion protein comprising an activation domain and a DNA binding domain. In a preferred embodiment, the activation domain is derived from an organism selected from the group consisting of virus and eukaryotic organism. In a more preferred embodiment, the virus is a herpes simplex virus. In a yet more preferred embodiment, the activation domain is VP16. In an alternative more preferred embodiment, the eukaryotic organism is human. In an even more preferred embodiment, the activation domain is B42. In a yet alternative more preferred embodiment, the eukaryotic organism is yeast. In an even more preferred embodiment, the activation domain is GAL4. In an alternative preferred embodiment, the DNA binding domain is derived from a protein wherein the protein is derived from a prokaryotic organism. In a more preferred embodiment, the prokaryotic organism is *Escherichia coli*. In an even more preferred embodiment, the protein is selected from the group consisting of ArsR, AscG, CscR, DeoR, DgoR, FruR, GalR, GatR, LexA, CI, LacI, RafR, and TetR.

The invention addtionally provides a composition comprising a first construct, second construct and third construct, wherein: (a) the first construct comprises a first promoter operably linked to a first gene, wherein the first promoter is inducible, and the first gene encodes a first protein; and (b) the second construct comprises a second promoter operably linked to a second gene, to a third gene, and to a first gene of interest, wherein the second promoter is constitutive, the second gene encodes a second protein, the third gene encodes a third protein, and the first gene of interest encodes a first protein of interest; (c) the third

construct comprises a third promoter operably linked to a fourth gene, wherein the third promoter is inducible and wherein the fourth gene encodes a fourth protein, wherein: (i) the first promoter is capable of binding to: (1) the second protein wherein the binding represses transcription of the first gene; and (2) the fourth protein wherein the binding increases transcription of the first gene and increases the strength of the first promoter; (ii) the second promoter is capable of binding to the first protein and wherein the binding represses transcription of the second gene and of the first protein of interest; and (iii) the third promoter is capable of binding to the third protein wherein the binding increases transcription of the fourth gene. In one embodiment, the first construct further comprises a second gene of interest operably linked to the first promoter and wherein the second gene of interest encodes a second protein of interest.

Also provided by the invention is a composition comprising a first construct, second construct and third construct, wherein: (a) the first construct comprises a first promoter operably linked to a first gene, a second gene, and to a first gene of interest, wherein the first promoter is inducible, the first gene encodes a first protein, the second gene encodes a second protein, and the first gene of interest encodes a first protein of interest; and (b) the second construct comprises a second promoter operably linked to a third gene, wherein the second promoter is constitutive and the third gene encodes a third protein; (c) the third construct comprises a third promoter operably linked to a fourth gene wherein the fourth gene encodes a fourth protein, wherein: (i) the first promoter is capable of binding to: (1) the third protein wherein the binding represses transcription of the first gene, the second gene, and of the first gene of interest; and (2) the fourth protein wherein the binding increases transcription from the first promoter and increases the strength of the first promoter; (ii) the second promoter is capable of binding to the first protein wherein the binding represses transcription of the third gene; and (iii) the third promoter is capable of binding to the second protein wherein the binding reduces transcription of the fourth gene.

In one embodiment, the second construct further comprises a second gene of interest operably linked to the second promoter and wherein the second gene of interest encodes a second protein of interest.

In an alternative embodiment, at least one of the first and second promoters is derived from a prokaryotic organism. In a preferred embodiment, the prokaryotic organism is *Escherichia coli*. In a more preferred embodiment, the inducible first promoter is derived from a nucleic acid sequence selected from the group consisting of arabanose operon, CAD

operon, deoP2, Cyn operon, Dsd operon, formate dehydrogenase/hydrogenase operon, malPp, monoamine oxidase operon, IlvC operon, and urease operon. In an alternative more preferred embodiment, the constitutive second promoter is derived from a nucleic acid sequence selected from the group consisting of P_L promoter, P_{trc} promoter, arsenic operon, ASC operon, sucrose operon, deoxyribose operon, DGORKAT operon, fructose operon, galactose operon, galactitol operon, SOS response regulon, raffinose operon, and tetracycline resistance operon.

In another embodiment, the first and second promoters is derived from a virus. In a preferred embodiment, the virus is selected from the group consisting of human cytomegaolvirus, herpes simplex virus, and simian virus 40. In a more preferred embodiment, the constitutive second promoter is selected from the group consisting of P_{hCMV}, P_{HSVtk}, and P_{SV40}.

In yet another embodiment, at least one of the first and second promoter is derived from a eukaryotic organism. In a preferred embodiment, the eukaryotic organism is selected from the group consisting of human and yeast.

In yet an further embodiment, the fourth gene is derived from a prokaryotic organism. In a preferred embodiment, the prokaryotic organism is *Escherichia coli*. In a more preferred embodiment, the fourth gene encodes a protein selected from the group consisting of AraC, CadC, CRP, CynR, DsdC, FhlA, MalT, MaoB, IlvY, and UreR.

In still another embodiment, the third gene is derived from a prokaryotic organism. In a preferred embodiment, the prokaryotic organism is *Escherichia coli*. In a more preferred embodiment, the third gene encodes a protein selected from the group consisting of ArsR, AscG, CscR, DeoR, DgoR, FruR, GalR, GatR, LexA, CI, LacI, RafR, and TetR.

In yet another embodiment, the third promoter is derived from a prokaryotic organism. In a preferred embodiment, the prokaryotic organism is *Escherichia coli*. In a more preferred embodiment, the third promoter is derived from a nucleic acid sequence selected from the group consisting of P_L promoter, P_{trc} promoter, arsenic operon, ASC operon, sucrose operon, deoxyribose operon, DGORKAT operon, fructose operon, galactose operon, galactitol operon, SOS response regulon, raffinose operon, and tetracycline resistance operon.

In a further embodiment, the third promoter is derived from a eukaryotic organism. In an alternative further embodiment, the third promoter is derived from a virus. In a preferred embodiment, the virus is selected from the group consisting of human

cytomegalovirus, herpes simplex virus and simian virus 40. In more preferred embodiment, the viral third promoter is selected from the group consisting of P_{hCMV}, P_{HSVtk}, and P_{SV40}.

In yet another embodiment, at least the inducible first promoter is operably linked to a first operator sequence, the consitutive second promoter is operably linked to a second operator sequence, or the constitutive third promoter is operably linked to a third operator sequenc, and wherein the constitutive second and third promoters and the inducible first promoter are derived from a first organism, and the first, second and third operator sequences are derived from a second organism. In a preferred embodiment, the first and second organisms are the same. In a more preferred embodiment, the first and second organisms are prokaryotic organisms. In a yet more preferred embodiment, the first and second prokaryotic organisms are *Escherichia coli*. In an alternative more preferred embodiment, the first and second organisms are eukaryotic organisms. In an alternative preferred embodiment, the first and second organisms are different. In a more preferred embodiment, at least one of the first and second organisms is a prokaryotic organism. In a yet more preferred embodiment, the prokaryotic organism is *Escherichia coli*. In an alternative more preferred embodiment, at least one of the first and second organisms is a eukaryotic cell. In another alternative preferred embodiment, the first organism is a virus, and the second organism is a prokaryotic organism. In a more preferred embodiment, the prokaryotic organism is *Escherichia coli*. In a yet more preferred embodiment, the operator sequence is selected from the group consisting of O_{lac}, O_{lex}, O_{tet1}, O_{tet2}, O_{R1}, O_{R2}, O_{R3}, O_{L1}, O_{L2} and O_{L3}. In aln alternative more preferred embodiment, the virus is selected from the group consisting of human cytomegalovirus, herpes simplex virus and simian virus 40. In an alternative yet more preferred embodiment, the inducible first promoter is derived from a nucleic acid sequence selected from the group consisting of arabanose operon, CAD operon, deoP2, Cyn operon, Dsd operon, formate dehydrogenase/hydrogenase genes, malPp, monoamine oxidase gene, IlvC gene, and urease operon. In another alternative yet more preferred embodiment, the constitutive second promoter is derived from a nucleic acid sequence selected from the group consisting of P_L promoter, P_{trc} promoter, arsenic operon, ASC operon, sucrose operon, deoxyribose operon, DGORKAT operon, fructose operon, galactose operon, galactitol operon, SOS response regulon, raffinose operon, and tetracycline resistance operon. In an alternative more preferred embodiment, the viral promoter is selected from the group consisting of P_{hCMV}, P_{HSVtk}, and P_{SV40}.

In yet another embodiment, the fourth protein is a fusion protein comprising an activation domain and a DNA binding domain. In a preferred embodiment, the activation domain is derived from an organism selected from the group consisting of virus and eukaryotic organism. In a more preferred embodiment, the virus is a herpes simplex virus. In a yet more preferred embodiment, the activation domain is VP16. In an alternative more preferred embodiment, the eukaryotic organism is human. In a yet more preferred embodiment, the activation domain is B42. In another alternative more preferred embodiment, the eukaryotic organism is yeast. In a more preferred embodiment, the activation domain is GAL4. In an alternative preferred embodiment, the DNA binding domain is derived from a protein wherein the protein is derived from a prokaryotic organism. In a more preferred embodiment, the prokaryotic organism is *Escherichia coli*. In an even more preferred embodiment, the protein is selected from the group consisting of ArsR, AscG, CscR, DeoR, DgoR, FruR, GalR, GatR, LexA, CI, LacI, RafR, and TetR.

Also provided by the invention is a composition comprising a first construct, second construct and third construct, wherein: (a) the first construct comprises a first promoter operably linked to a first gene and to a first gene of interest, wherein the first promoter is inducible, the first gene encodes a first protein and the first gene of interest encodes a first protein of interest; (b) the second construct comprises a second promoter operably linked to a second gene, wherein the second promoter is constitutive and second gene encodes a second protein; and (c) the third construct comprises a third promoter operably linked to a third gene wherein the third gene encodes a third protein, wherein: (i) the first promoter is capable of binding to: (1) the second protein wherein the binding reressses transcription of the first gene and of the first gene of interest; and (2) the third protein wherein the binding increases transcription from the first promoter and increases the strength of the first promoter; (ii) the second promoter is capable of binding to the first protein wherein the binding represses transcription of the second gene; and (iii) the third promoter is capable of binding to the first protein wherein the binding reduces transcription of the third gene.

The invention additionally provides a composition comprising a first construct, second construct and third construct, wherein: (a) the first construct comprises a first promoter operably linked to a first gene, and a second gene, wherein the first promoter is inducible, the first gene encodes a first protein, and the second gene encodes a second protein; and (b) the second construct comprises a second promoter operably linked to a third gene and to a first gene of interest, wherein the second promoter is constitutive, the third

gene encodes a third protein, and the first gene of interest encodes a first protein of interest; (c) the third construct comprises a third promoter operably linked to a fourth gene wherein the fourth gene encodes a fourth protein, wherein: (i) the first promoter is capable of binding to: (1) the third protein wherein the binding represses transcription of the first gene and of the second gene; and (2) the fourth protein wherein the binding increases transcription from the first promoter and increases the strength of the first promoter; (ii) the second promoter is capable of binding to the first protein wherein the binding represses transcription of the third gene and of the first gene of interest; and (iii) the third promoter is capable of binding to the second protein wherein the binding reduces transcription of the fourth gene. In one embodiment, the first construct further comprises a second gene of interest operably linked to the first promoter and wherein the second gene of interest encodes a second protein of interest.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic of one embodiment of a toggle switch construct.

Figure 2 is a phase plane diagram of Equations 1 which describe the behavior of a toggle switch construct. A: A bistable toggle network with balanced promoter strengths. B: A monostable toggle network with imbalanced promoter strengths.

Figure 3 is a diagram showing a bifurcation analysis of the toggle switch construct.

Figure 4 is a schematic of an exemplary embodiment of a toggle switch construct.

Figure 5 is a schematic of one embodiment of an adjustable-threshold switch construct.

Figure 6 is a phase plane diagram of Equations 3 for the adjustable-threshold switch construct.

Figure 7 is a diagram showing the structure of the threshold in the adjustable-threshold switch construct.

Figure 8 is a bifurcation diagram showing the size of the hysteresis and the location of the threshold for a range of values of promoter strengths in an adjustable-threshold switch construct.

Figure 9 is a schematic of alternative embodiments of a two-state oscillator construct.

Figure 10 is a diagram showing simulation of the two-state oscillator construct. (A) Time-delay: $\kappa_d = 5$, (B) Time-delay: $\kappa_d = 10$, (C) $\tau_d = 8$, (D) $\tau_d = 16$.

Figure 11 is a map of the pTAK1 plasmid carrying an exemplary toggle switch construct.

Figure 12 shows the nucleic acid sequence of (A) P_{trc} promoter (SEQ ID NO:1) and (B) P_L promoter (SEQ ID NO:2).

Figure 13 is an outline of the protocol for constructing the pOS_1 and pOS_1-GFPuv plasmids.

Figure 14 is an outline of the protocol for constructing the pOS_2 and pOS_2-GFPuv plasmids.

Figure 15 is an outline of the protocol for constructing the $pTAK_1$ plasmid using the pOS_1 and pOS_2 plasmids.

Figure 16 is a schematic of prokaryotic and eukaryotic toggle switch constructs.

Figure 17 is an exemplary eukaryotic toggle switch construct.

Figure 18 is a schematic of a eukaryotic adjustable-threshold switch construct.

Figure 19 is an exemplary eukaryotic adjustable-threshold switch construct.

Figure 20 is a schematic representation of an alternative toggle switch design. Repressor 1 inhibits transcription from Promoter 1 and is induced by Inducer 1. Repressor 2 inhibits transcription from Promoter 2 and is induced by Inducer 2.

Figure 21 is a map of the four plasmid types used in the construction of the toggle.

Figure 22 shows the nucleic acid sequence of promoters (A) and ribosome binding sites (B) used to construct the toggle plasmids.

Figure 23 is a graph demonstrating bistability. Arrowheads indicate times of sampling and dilution of cells into fresh medium. a: Class 1 toggles and controls. b: Class 2 toggles and controls. c: Long-term test of pTAK117 bistability. Cells were initially divided, diluted and induced with IPTG for 6 hours (circles) or grown without inducer (squares).

Figure 24 is a diagram of the proposed structure of the bistable regions for the class 1 and class 2 toggles.

Figure 25 is a diagram showing the toggle switch induction threshold. a: Steady-state gene expression after 17 hour induction. b: Fraction of toggle cells in the high state at various concentrations of IPTG. c: Scatter plots (left plots) and histograms (right plots) illustrating the condition of the toggle cells at points 2, 3 and 4 (of panel a) near the bifurcation point.

Figure 26 is a graph showing pTAK117 switching time.

Figure 27 shows (A) a schematic representation of an exemplary eukaryotic adjustable-threshold switch construct, and (B) the nucleic acid sequence of the P_{bad} promoter which is fused to the O_{lac} operator region of P_{trc}.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

The terms "gene of interest," "nucleotide sequence of interest," and "protein of interest" refer to any gene, nucleotide sequence, and protein, respectively, the manipulation of which may be deemed desirable for any reason by one of ordinary skill in the art (*e.g.*, confer improved qualities). For example, nucleotide sequences of interest include, but are not limited to, coding sequences of structural genes (*e.g.*, reporter genes, selection marker genes, oncogenes, drug resistance genes, growth factors, *etc.*), and of regulatory genes (*e.g.*, activator protein 1 (AP1), activator protein 2 (AP2), Sp1, *etc.*).

As used herein, the term "gene" means the deoxyribonucleotide sequences comprising the coding region of a structural gene and including sequences located adjacent to the coding region on both the 5' and 3' ends such that the gene corresponds to the length of the full-length mRNA. The sequences which are located 5' of the coding region and which are present on the mRNA are referred to as 5' non-translated sequences. The sequences which are located 3' or downstream of the coding region and which are present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene which are transcribed into heterogenous nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide. In addition to containing introns, genomic forms of a gene may also include sequences located on both the 5' and 3' end of the sequences which are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5' flanking region may contain regulatory sequences such as promoters and enhancers which control or influence the transcription of the gene. The 3' flanking region

may contain sequences which direct the termination of transcription, posttranscriptional cleavage and polyadenylation.

As used herein, the term "regulatory element" refers to a genetic element which controls some aspect of the expression of nucleic acid sequences. For example, a promoter is a regulatory element which facilitates the initiation of transcription of an operably linked coding region. Other regulatory elements are splicing signals, polyadenylation signals, termination signals, *etc.*

The terms "promoter" and "promoter sequence" as used herein interchangeably refer to a region of a gene at which initiation and rate of transcription are controlled. Promoters generally contain the site at which RNA polymerase binds and also sites for the binding of regulatory proteins (*e.g.*, repressors, transcription factors). As used herein, the term "promoter sequence" refers to a single promoter sequence as well as to a plurality (*i.e.*, one or more) of promoter sequences which are operably linked to each other. For example, one of skill in the art knows that it may be desirable to use a double promoter sequence (*i.e.*, a DNA sequence containing two promoter sequences) or a triple promoter sequence (*i.e.*, a DNA sequence containing three promoter sequences) to control expression of a DNA sequence of interest. Promoters contemplated to be within the scope of the invention include "cytoplasmic promoters" and "nuclear promoters." A "cytoplasmic promoter" as used herein refers to a promoter which is recognizable by an RNA polymerase that is located in the cytoplasm. Cytoplasmic promoters are exemplified by the T_7 promoter and the SP6 promoter. The term "nuclear promoter" as used herein refers to a promoter which is recognizable by an RNA polymerase that is located in the nucleus. Examples of nuclear promoters include the va RNA I promoter and the promoters for RNA polymerases I, II, and III. The activity of cytoplasmic and nuclear promoters may be determined using methods known in the art, *e.g.*, Northern blot analysis. The location of a promoter in a gene may be identified by approaches and methods well known in the art, including DNase foot printing of the RNA polymerase-bound genome DNA, mutational analysis, *etc.* Alternatively, where the sequence of a cognate promoter is known, the promoter sequence may be synthesized.

Promoters may be constitutive or inducible. The term "constitutive promoter" refers to a promoter whose activity does not quantitatively increase in response to a specific molecule. The term "constitutive promoter" more preferably refers to a promoter whose activity (i.e., ability to initiate transcription of a DNA sequence into mRNA) is above background level or detectable by Northern blot hybridization in the absence of a specific

regulatory molecule. In contrast, an "inducible promoter" is a promoter whose activity may be quantitatively increased in response to a specific molecule (*e.g.*, enhancer protein). The activity of both constitutive promoters and inducible promoters may, however, be quantitatively decreased in response to a specific molecule (*e.g.*, a repressor protein).

The terms "cognate promoter" and "associated promoter" when made in reference to a protein (*e.g.*, repressor protein, enhancer protein, *etc.*) refer to a promoter sequence which binds to the protein, wherein such binding results in a change (*i.e.*, a decrease or increase) in the promoter activity as compared to the promoter activity in the absence of the protein and as measured by, for example, Northern blot analysis.

The terms "operator" and "operator sequence" refer to a DNA sequence which is generally located in the vicinity of a promoter and to which a repressor protein binds to modulate (generally decrease) transcription of that gene or operon. Operators may be located upstream or downstream of apromoter and within the coding region of a gene.

The terms "operably linked," "in operable combination", and "in operable order" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

A "repressor protein" is a protein which binds specifically to an operator sequence adjacent to a promoter and reduces the level of transcription of a gene or operon which is operably linked to the promoter as compared to the level of transcription of the gene or operon in the absence of the repressor protein.

An "activator protein" is a protein which binds to a promoter and increases the level of transcription of a gene or operon which is operably linked to the promoter relative to the level of transcription of the gene or operon in the absence of the activator protein.

The terms "repress transcription," "reduce transcription," "decrease transcription," "inhibit transcription" and grammatical equivalents thereof when made in reference to transcription of a gene in response to a stimulus (*e.g.*, a protein) refer to a reduction in the quantity of transcribed gene (as measured by, for example, Northern blot analysis) in the presence of the protein as compared to the quantity of transcribed gene in the absence of the protein; the quantity of the transcribed gene in the presence of the protein is preferably less than 50%, more preferably less than 25%, and most preferably less than 10% of the quantity of the transcribed gene in the absence of the protein. A "reduced quantity of transcribed

gene" includes a quantity at the background level of, or is undetectable by, Northern blot hybridization. When a background level or undetectable level of transcribed gene is measured, this may indicate that the gene is not transcribed. A "reduced quantity of transcribed gene" need not, although it may, mean an absolute absence of transcription of the gene. The invention does not require, and is not limited to, constructs in which gene transcription is 100% ablated.

The terms "induce transcription" and "increase transcription" when made in reference to transcription of a gene in response to a stimulus (*e.g.*, a protein) refer to an increase in the level of transcription of the gene in the presence of the protein relative to the level of transcription of the gene in the absence of the protein.

The term "altering transcription" when made in reference to a gene of interest refers to a change (*i.e.*, decrease or increase) in the level of transcription of the gene of interest.

The terms "repress a promoter," "inhibit a promoter" and grammatical equivalents thereof when made in reference to the effect of an agent on a promoter mean that the agent represses transcription of a gene which is under the control of the promoter. The terms "induce a promoter" and "activate a promoter" and grammatical equivalents thereof when made in reference to the effect of an agent on a promoter mean that the agent induces transcription of a gene which is under the control of the promoter.

The term "expression vector" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in prokaryotes include a promoter, optionally an operator sequence, a ribosome binding site and possibly other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals. Where efficient expression of recombinant DNA sequences in eukaryotic cells is desired, it is preferred (though not required) that signals which direct the efficient termination and polyadenylation of the resulting transcript are used. Transcription termination signals are generally found downstream of the polyadenylation signal and are a few hundred nucleotides in length. The term "poly A site" or "poly A sequence" as used herein denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the recombinant transcript is desirable as transcripts lacking a poly A tail are unstable and are rapidly degraded. The poly A signal utilized in an expression vector may be "heterologous" or "endogenous." An endogenous poly A signal is

one that is found naturally at the 3' end of the coding region of a given gene in the genome. A heterologous poly A signal is one which is isolated from one gene and placed 3' of another gene. A commonly used heterologous poly A signal is the SV40 poly A signal. The SV40 poly A signal is contained on a 237 bp *Bam*HI/*Bcl*I restriction fragment and directs both termination and polyadenylation. This 237 bp fragment is contained within a 671 bp *Bam*HI/*Pst*I restriction fragment.

The term "periodic expression" when made in reference to a gene refers to a temporal change in the level of expression of the gene as determined by, for example, Western blot analysis; the level of expression of the gene at a given time is different from the level of expression of the gene at a different time.

The term "transfection" as used herein refers to the introduction of a transgene into a cell. The term "transgene" as used herein refers to any nucleic acid sequence which is introduced into a cell by experimental manipulations. A transgene may be an "endogenous DNA sequence," or a "heterologous DNA sequence" (*i.e.*, "foreign DNA"). The term "endogenous DNA sequence" refers to a nucleotide sequence which is naturally found in the cell into which it is introduced so long as it does not contain some modification (*e.g.*, a point mutation, the presence of a selectable marker gene, *etc.*) relative to the naturally-occurring sequence. The term "heterologous DNA sequence" refers to a nucleotide sequence which is not endogenous to the cell into which it is introduced. Heterologous DNA includes a nucleotide sequence which is ligated to, or is manipulated to become ligated to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Heterologous DNA also includes a nucleotide sequence which is naturally found in the cell into which it is introduced and which contains some modification relative to the naturally-occurring sequence.

Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, biolistics (*i.e.*, particle bombardment) and the like.

Transfection may be stable or transient. The term "stable transfection" or "stably transfected" refers to the introduction and integration of a transgene into the genome of the transfected cell. The term "stable transfectant" refers to a cell which has stably integrated one or more transgenes into the genomic DNA. The term "transient transfection" or "transiently transfected" refers to the introduction of one or more transgenes into a

transfected cell in the absence of integration of the transgene into the host cell's genome. The term "transient transfectant" refers to a cell which has transiently integrated one or more transgenes.

The term "deletion" is defined as a change in the nucleotide sequence in which one or more nucleotides are absent. An "insertion" or "addition" is that change in a nucleotide sequence which has resulted in the addition of one or more nucleotides as compared to, for example, the naturally occurring sequence. A "substitution" results from the replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

The term "mismatch" refers to a non-covalent interaction between two nucleic acids, each nucleic acid residing on a different polynucleic acid sequence, which does not follow the base-pairing rules. For example, for the partially complementary sequences 5'-AGT-3' and 5'-AAT-3', a G-A mismatch is present.

The term "strand break" when made in reference to a double stranded nucleic acid sequence includes a single-strand break and/or a double-strand break. A single-strand break refers to an interruption in one of the two strands of the double stranded nucleic acid sequence. This is in contrast to a double-strand break which refers to an interruption in both strands of the double stranded nucleic acid sequence. Strand breaks may be introduced into a double stranded nucleic acid sequence either directly (e.g., by ionizing radiation) or indirectly (e.g., by enzymatic incision at a nucleic acid base).

The terms "nucleic acid" and "unmodified nucleic acid" as used herein refers to any one of the known five nucleic acid bases (*i.e.*, guanine, adenine, cytosine, thymine and uracil). The term "modified nucleic acid" refers to a chemically modified nucleic acid. Illustrative of such modifications would be replacement covalent modifications of the bases, for example alkylation of amino and ring nitrogens as well as saturation of double bonds.

The term "homo-multimer" as used herein in reference to a polypeptide refers to a composition which contains two or more polypeptides, where the amino acid sequence of each and every polypeptide is the same, and where the polypeptides interact non-covalently. A homo-multimer may be a homo-dimer, homo-trimer, homo-tetramer, *etc.* Homo-dimers contain two polypeptides, homo-trimers contain three polypeptides, *etc.* In contrast, the term "hetero-multimer" when used in reference to a polypeptide refers to a composition which contains two or more polypeptides, where the amino acid sequence of at least one polypeptide is different from the amino acid sequence of another polypeptide, and where the

polypeptides interact via non-covalent bonds. A hetero-multimer may be a hetero-dimer, hetero-trimer, hetero-tetramer, *etc.*

The term "activation domain" when made in reference to a protein refers to a polypeptide sequence which is capable of binding to RNA polymerase and of increasing transcription of a gene or operon which is operably linked to the target promoter of the activation domain. Activation domains in proteins may be determined by a variety of means known in the art including, but not limited to, X-ray crystallography, nuclear magnetic resonance, yeast two-hybrid assays, site-directed mutagenesis and column chromatography. Functional recombinant activation domains may be made by fusing a DNA sequence encoding the activation domain to a DNA sequence encoding a DNA binding domain.

The term "DNA binding domain" as used herein in reference to a protein refers to a polypeptide sequence which is capable of binding to a DNA sequence. DNA binding domains may be determined by a variety of means known in the art including, for example, electrophoretic mobility shift assays, chemical and enzymatic DNA footprinting, yeast two-hybrid assays, site-directed mutagenesis column chromatography, phage display, and DNA chip screening.

The term "transient" when made in reference to exposing a cell to an agent to bring about a change in the level of transcription of a gene of interest in the cell refers to exposing the cell for a period of time which is sufficient to bring about a change in the quantity of transcribed gene of interest (as measured by, for example, Northern blot analysis) in the presence of the agent as compared to the quantity of transcribed gene of interest in the absence of the agent.

The terms "expressed periodically," "periodic expression" and grammatical equivalents thereof when made in reference to a gene refer to a temporal change in the quantity of the transcribed gene. A temporal change includes an increase in the quantity of transcribed gene from a first to a second quantity followed by a decrease from the second quantity to a third quantity which is substantially similar to the first quantity (*i.e.*, preferably at least 60% of the first quantity, more preferably at least 80% of the first quantity, and most preferably at least 90% of the first quantity) of transcribed gene. Temporal changes also include a decrease in the quantity of transcribed gene from a first to a second quantity followed by an increase from the second quantity to a third quantity which is substantially similar to the first quantity (*i.e.*, preferably at least 60% of the first quantity, more

preferably at least 80% of the first quantity, and most preferably at least 90% of the first quantity) of transcribed gene.

DESCRIPTION OF THE INVENTION

The present invention provides genetic applets, *i.e.*, a network of interacting genes which may be delivered into cells to execute a sequence of cellular functions while responding to intracellular and extracellular signals. The methods and constructs provided by this invention extend the functionality of synthetic gene regulatory systems beyond that of currently available systems. The behaviors produced by the constructs provided by this invention, including multistability, threshold-based switching, and oscillatory expression, form the basis for autoregulated, synthetic gene circuits. Furthermore, the constructs, by themselves, have immediate application in gene therapy, tissue engineering, biotechnology and biocomputing. In addition, these constructs serve as means to develop a predictive theory of gene expression.

Specifically, the genetic applets provided herein are exemplified by toggle switch constructs, adjustable-threshold switch constructs and multi-state oscillator constructs. Genetic applets provide a model for gene networks which have applications in clinical therapy, biomedical research, and biotechnology. For example, genetic applets may be used as sensitive *in vivo* detectors of chemical or biological warfare agents, allow precisely controlled gene expression in gene therapies, and provide a mechanism for initiating apoptosis in engineered micro-organisms.

Because their components are amenable to manipulation, genetic applets also serve as models for the regulation of gene expression. Gene expression in cells is a complex process composed of thousands of interacting genes that coordinate everything from embryonic development to cell differentiation to immune responses. While advances in molecular biology and bioinformatics have revealed the identity of many of these regulatory genes, they have not clarified how these genes interact to produce the observed cellular behaviors. In contrast to prior art approaches which rely on reverse engineering biological systems for studying gene function, the genetic applets provided herein employ a forward engineering approach. This approach has the advantage that it permits the complete manipulation of each component in the system, thus allowing the complexities of natural gene networks to be "engineered out" of the applets and providing highly simplified, highly controlled "models" of natural gene networks.

The invention is further described under (A) Design Principles of Genetic Applets, and (B) Construction of Genetic Applets.

A. Design Principles of Genetic Applets

The forward engineering approach to designing genetic applets employs mathematical and computer modeling which integrates nonlinear dynamics, chemical physics, biochemistry, and molecular biology. Mathematical and computer modeling of biological systems has been found to be predictive of the behavior of recombinant constructs, such as cross-regulation constructs [Chen *et al.* (1993) Gene 180:15-22; Chen *et al.* (1995) Biotechnol. Prog. (US) 11(4):397-402; Bailey *et al.*, U.S. Patent No. 5,416,008, the entire contents of which are herein incorporated by reference]. The design principles of the invention's constructs are further described below.

1. Genetic Toggle Switches

The toggle switch constructs provided herein contain two mutually inhibitory genes as depicted in Figures 1 and 20. Promoter 1, P_1, efficiently transcribes *Gene 1* unless inhibited by the repressor protein encoded by *Gene 2*. Promoter 2, P_2, efficiently transcribes *Gene 2* unless inhibited by the repressor protein encoded by *Gene 1*. Open arrows indicate direction of transcription. *Clone* is an additional gene or genes which may be placed under the control of P_1 or P_2. The first operon (*i.e.*, P_1-*Gene 1-Clone*) and second operon (*i.e.*, P_2-*Gene 2*) may be on the same or on different vectors. Furthermore, the location of *Gene 1* and *Clone* in relation to P_1 may be reversed (*i.e.*, such that the operon is P_1-*Clone-Gene 1*) so long as each of *Gene 1* and *Clone* are operably linked to P_1. One of skill in the art appreciates that in addition to the first *Clone* which is under the control of P_1, a second *Clone* (which is the same or different from the first *Clone*) may be placed under the control of P_2.

In Figures 1 and 20, the first and second genes are mutually inhibitory in that the protein product of the first gene (*i.e.*, the first repressor protein) represses transcription of the second gene, while the protein product of the second gene (*i.e.*, the second repressor protein) represses transcription of the first gene. Transcription by this system is characterized by its bi-stability, *i.e.*, there is no "default" transcription state since transcription is stable in either one of two transcription states. A bi-stable system may be distinguished from a mono-stable system by the system's response to a transient application

of an inducing agent. A bi-stable system that is switched from state 1 to state 2 by an inducing agent will remain in state 2 after the removal of the agent. In contrast, a mono-stable system that is switched from state 1 to state 2 by an inducing agent will return to state 1 after removal of the inducing agent. The bi-stable switch can be returned to state 1 from state 2 by the transient application of a second agent. In state 1, there is transcription from the first promoter and suppression of transcription from the second promoter. In state 2, there is suppression of transcription from the first promoter and transcription from the second promoter. The system is also characterized by being able to switch from state 1 to state 2, and back to state 1. This back-and-forth "on-off" switching may be brought about by a transient application of one or more activating agents (*e.g.*, UV light, a chemical, a protein, temperature shift, *etc.*.) which inhibits the function of the first or second repressor proteins to cause the induction of the "off" gene and, subsequently, the repression of the "on" gene.

Thus, a construct containing a first gene under the control of a first promoter and a second gene under the control of a second promoter where the transcription product of the first gene represses the second promoter and the transcription product of the second gene represses the first promoter, can be either bi-stable or monostable. The term "bi-stable" and grammatical equivalents thereof means that the construct is capable of expressing either the first or second genes, that transient application of an agent which reduces repression of the first promoter results in transcription of the first gene and in the repression of transcription of the second gene even after removal of the agent, and that transient application of another agent which reduces repression of the second promoter results in transcription of the second gene and in the repression of transcription of the first gene even after removal of the other agent. In contrast, the term "monostable" and grammatical equivalents thereof means that while the construct is capable of expressing either the first or second genes, the construct has a default expression state (*i.e.*, expression of the first gene or expression of the second gene) in the absence of stimuli which are external to the construct. Thus, where the default state is expression of the first gene, transient application of an agent which reduces repression of the second promoter results in transcription of the second gene and in the repression of transcription of the first gene. Transcription of the second gene continues so long as the agent is present. Removal of the agent results in a switch back to expression of the first gene in the substantial absence of expression of the second gene. Where the default state is expression of the second gene, transient application of an agent which reduces repression of

the first promoter results in transcription of the first gene and in the repression of transcription of the second gene. Transcription of the first gene continues so long as the agent is present. Removal of the agent results in a switch back to expression of the second gene and in the repression of expression of the first gene.

The design for the genetic toggle is based on mathematical models describing the dynamic interactions of two mutually inhibitory genes as exemplified in Figures 1 and 20. This system exhibits two stable states. In each state, only one of "*Gene 1-Clone*" and "Gene 2" is expressed by the host cell. The genetic toggle switch construct is modeled by the following two equations 1:

$$\frac{du}{dt} = \frac{k_1\lambda_1/\delta_1}{1 + K_{mu}\,(1 + v^{\gamma} / K_{iv}^{\gamma})} - d_1 u$$

$$\frac{dv}{dt} = \frac{k_2\lambda_2/\delta_2}{1 + K_{mv}\,(1 + u^{\beta} / K_{iu}^{\beta})} - d_1 v$$

where,

u = concentration of gene product 1,

v = concentration of gene product 2,

λ_1 = maximum rate of synthesis of gene 1 mRNA by RNA polymerase,

λ_2 = maximum rate of synthesis of gene 2 mRNA by RNA polymerase,

δ_1 = rate of degradation of gene 1 mRNA,

δ_2 = rate of degradation of gene 2 mRNA,

k_1 = rate of synthesis of gene product 1 by the ribosome,

k_2 = rate of synthesis of gene product 2 by the ribosome,

K_{mu} = Michaelis constant for RNAP binding and transcription of *Gene 1*,

K_{mv} = Michaelis constant for RNAP binding and transcription of *Gene_2*,

K_{iu} = equilibrium constant for inhibitory binding of gene product 1 to promoter 2,

K_{iv} = equilibrium constant for inhibitory binding of gene product 2 to promoter 1,

d_1 = rate of degradation of gene products 1 and 2,

β = cooperativity of binding of gene product 1,

γ = cooperativity of binding of gene product 2.

Equations 1 are based on the assumption that gene expression can be modeled using the law of mass action. Although gene expression does not typically involve a large number of particles, considerable evidence exists that such approximations provide a reasonable description of gene expression. For example, earlier work using a reconstituted enzyme system [Schellenberger *et al.*, Adv. Enzyme Regul. 19, 257-284 (1980)] demonstrated the effectiveness of nonlinear mathematics in predicting novel qualitative behaviors, including multistability and hysteresis, in biochemical reaction networks. In addition, a variety of physical and mathematical approaches, including logical (discrete) [Glass *et al.*, J. Theor. Biol. 54, 85-107 (1975); Glass & Kauffman, J. Theor. Biol. 39, 103-129 (1973); Kauffman, J. Theor. Biol. 44, 167-190 (1974); Thomas, J. Theor. Biol. 73, 631-656 (1978); Thomas, J. Theor. Biol. 153, 1-23 (1991)], piece-wise linear [Tchuraev, J. Theor. Biol. 151, 71-87 (1991)], nonlinear [Arkin & Ross, Biophys. J. 67, 560-578 (1994); Bhalla & Iyengar, Science 283, 381-387 (1999); Glass, J. Chem. Phys. 63, 1325-1335 (1975)], statistical-mechanical [Shea & Ackers, J. Mol. Biol. 181, 211-230 (1985); Smith *et al.*, Math. Biosci. 36, 61-86 (1977)] and stochastic [Arkin *et al.*, Genetics 149, 1633-1648 (1998); McAdams & Arkin, Proc. Natl. Acad. Sci. USA 94, 814-819 (1997); McAdams & Arkin, Annu. Rev. Biophys. Biomol. Struct. 27, 199-224 (1998)] formulations of the underlying biochemical dynamics, have had varying degrees of success in describing the behavior of gene networks.

The first term in each equation describes the synthesis of nascent proteins. Both transcription by the RNA polymerase and translation by the ribosome are included in the first term. Transcription, modeled with Michaelis-Menton kinetics, is competitively inhibited by the opposing gene product. Inhibition is achieved by the binding, as a homo-multimer, of one gene product to one or more sites in the opposing gene's promoter region. The multimeric interaction and the multiple binding sites are accounted for by the cooperativity exponents β and γ in the first term of each equation.

The second term describes the rate of degradation of proteins. In *E. coli*, the host cell for the inventors' toggle switch experiments, the dilution of proteins as a result of cell growth is the major determinant of the degradation rate. Since this rate is identical for all proteins in the cell, a single rate constant, d_1, is used in the model for protein degradation. However, the assumption of a single rate constant is not necessary for a functional toggle

switch. The bi-stable behavior will exist in the toggle with unequal degradation rates of the proteins, but a compensating adjustment in the promoter strengths, α_1 and α_2, may be necessary as described below. Additional assumptions, implicit in this model, are (i) mRNA turnover is rapid, and (ii) translation of each mRNA transcript occurs at its maximum rate, i.e. proteins are rapidly synthesized from the mRNA by an excess of ribosomes. These assumptions are supported by studies of transcription and translation [Alberts, B et al. (1994) Molecular Biology of the Cell, Garland Publishing, Inc., New York; Darnell, J et al. (1990) Molecular Cell Biology, Scientific American Books, Inc., New York].

Figure 2 shows the geometric structure of Equations 1. A cell with the toggle switch construct genes will settle to state 1 if its initial state is anywhere above the separatrix (*i.e.*, in the first basin of attraction); it will settle to state 2 if its initial state is anywhere below the separatrix (*i.e.*, in the second basin of attraction). Figure 2 reveals the origin of the bi-stability: the nullclines intersect in three places producing one unstable and two stable fixed points. From this figure, three key features of the system become apparent. First, the nullclines intersect three times, rather than once, because of their sigmoidal shape. The sigmoidal shape arises for $\beta, \gamma > 1$. Thus, the bi-stability of the system depends on the cooperative binding of the inhibitory proteins to the DNA. Second, the strengths of the promoters must be matched. The terms "matched" and "balanced" when made herein in reference to the strengths of a first and second promoters mean that the strengths of the first and second promoters are within the bi-stable region illustrated in Figure 3A. Promoter strength may be determined by quantitative assays of the expression of reporter genes such as the green fluorescent protein (GFP), β-galactosidase (β-gal), or chloramphenicol acetyl transferase (CAT). For example, in order to construct a first and second promoters with matched strengths, one of skill in the art knows to quantitatively determine the strength of each promoter using the same type of assay. If the strengths of the promoters do not fall within the bi-stable region illustrated in Figure 3A, one or both promoters may be modified and their strengths re-quantitated. Modification of one or both promoters may be repeated, if necessary, until the strengths of the promoters fall within the bi-stable region of Figure 3A. If the strengths are not matched, the nullclines will intersect only once producing a single stable fixed point. Third, the state of the toggle is switched by the application of a transient pulse of an inducing stimulus that pushes the system away from the stable steady state, over the separatrix, and into the opposite basin of attraction.

To build a working genetic toggle switch construct which produces robust bi-stable behavior *in vivo*, it is necessary to understand the effects of the eleven parameters in Equations 1. This analysis is facilitated by rescaling time and non-dimensionalizing the variables into the following Equations 2:

$$\frac{d\hat{u}}{d\tau} = \frac{\alpha_1}{1 + \hat{v}^{\beta}} - \hat{u}$$

$$\frac{d\hat{v}}{d\tau} = \frac{\alpha_2}{1 + \hat{u}^{\gamma}} - \hat{v} \qquad \textit{where,}$$

$$\tau = d_1\, t,$$

$$\hat{u} = \frac{u}{K_{iu}\,(1/K_{mv} + 1)^{1/\beta}},$$

$$\hat{v} = \frac{v}{K_{iv}\,(1/K_{mu} + 1)^{1/\gamma}},$$

$$\alpha_1 = \frac{k_1\lambda_1/\delta_1}{d_1\, K_{iu}\,(1 + K_{mu})(1/K_{mv} + 1)^{1/\beta}} \qquad \textit{and,}$$

$$\alpha_2 = \frac{k_2\lambda_2/\delta_2}{d_1\, K_{iv}\,(1 + K_{mv})(1/K_{mu} + 1)^{1/\gamma}}.$$

Nine parameters in Equations 1 collapse into two. Thus, the range of dynamic behaviors that can be produced by this system is easily understood by analysis of only four parameters. The two new parameters, α_1 and α_2, are the effective strength of promoters 1 and 2, respectively. As used herein, the terms "effective promoter strength" and "promoter strength" when used in reference to a nucleic acid sequence are interchangeably used to refer to the ability of the nucleic acid sequence to initiate transcription of an oligonucleotide sequence into mRNA and translation into protein. Promoter strength may be experimentally determined using methods known in the art, for example, Northern blots RNAase protection assays, reporter gene expression and SDS gel electrohproresis. Promoter strength is the net effect of the RNA polymerase (RNAP) binding affinity, the transcription rate, the inhibitor binding affinity and the translation efficiency and mRNA degradation rate. These physical quantities can be manipulated in the experimental system to achieve the desired promoter strength as further described below.

Figure 3 shows the result of two-parameter bifurcation analyses of the system. It is clearly seen in Figure 3A that the region of bi-stability grows larger as the strength of both

promoters is increased; thus, the system becomes more robust. In other words, as the absolute strengths of the promoters increase, the system will exhibit bi-stable behavior for larger relative imbalances in their strength. In Figure 3D, the bifurcation analysis reveals that the slopes of the bifurcation lines, for α_1 and α_2 are determined by β and γ; if both β and γ are less than or equal to 1, then bi-stability is unattainable. Calculations show that in order to achieve bi-stability, regardless of the value of β and γ, α_1 and α_2 cannot be less than 1. Calculations also show that if one promoter is too weak or too strong, then the system falls outside the bi-stable region in Figure 3C. Figure 3D shows that there is a "trade-off" between the values of β and γ on the one hand and the values of α_1 and α_2 on the other hand; as the values for β and γ increase, stability is attainable for decreased values of α_1 and α_2. Thus, to obtain bi-stability, at least one of the inhibitors must repress expression with cooperativity greater than one. This suggests that repressor multimerization, or multiple operator sites in the promoter, is necessary to obtain bi-stability. Higher-order multimerization will increase the robustness of the system, allowing weaker promoters to achieve bi-stability. The robustness of a system refers to its ability to exhibit the desired behavior under non-ideal conditions and unintended perturbations, *e.g.*, thermal fluctuations, mismatched promoter strengths, external agents that interfere with protein function or internal perturbations to gene expression such as DNA replication. On the other hand, the system will never exhibit bi-stability if the inhibitors bind non-cooperatively. Cooperativity is an inherent property of the protein repressor. Cooperative binding can arise through multimerization of the protein and through multiple binding sites in the promoter. Protein repressors which exhibit the desired degree of cooperativity are known in the art (e.g., those listed in the protein database SwissProt).

Furthermore, in one embodiment, if one promoter is too weak (e.g., α_1 or $\alpha_2 < 2$ for $\beta = \gamma = 2$), then bistability is unattainable regardless of the strength of the opposing promoter. In another embodiment, the bifurcation analysis reveals that the inhibitors must bind the DNA as dimers (*i.e.*, $\beta=\gamma=2$) for $\alpha_1=\alpha_2= 10$ (Figure 3B).

In other words, based on the inventors' preliminary determination, where the first and second repressor proteins bind as homo-dimers, the bi-stability of the toggle switch construct requires that the strengths of the first and second promoters be manipulated such that the strength of each of the first and second promoters (*i.e.*, α_1 and α_2) have a value of greater than 2. However, where the first and second repressor proteins bind as homo-multimers other than dimers, the minimum permissible value for α_1 or α_2 is reduced. This minimum

approaches a value of 1 as the degree of multimerization increases, but it never falls below 1. The strengths of the first and second promoters may be adjusted by manipulating RNAP binding, transcription elongation, inhibitor/activator binding, translation rate, and/or protein stability as described below.

While the above theory is described for an exemplary system with a competitive DNA-binding inhibitor, the same theory applies equally to systems with other types of inhibition. For example, inhibition through protein-protein binding, un-competitive, and non-competitive interactions will result in the same qualitative features of bi-stability.

The toggle switch constructs of the invention are exemplified by the construct depicted in Figure 4 in which the *lacI-Clone* genes are under the transcriptional control of the P_L promoter (which is derived from bacteriophage λ), and the cI gene is under the control of the P_{trc} promoter (which is a fusion of promoters derived from the Lac operon and the tryptophan operon). In this embodiment, the LacI repressor represses transcription by the P_{trc} promoter while the CI repressor represses transcription by the P_L promoter. If the system is initially expressing the *lacI-Clone*, then transient application of IPTG results in the formation of an IPTG-LacI complex which results in an increase in the levels of expressed CI repressor and consequently a decrease in the transcription activity of the P_L promoter which, in turn, results in a decrease in LacI repressor levels such that the construct switches the expression of the *cI* gene "on" and the *lacI-Clone* genes "off" even after the removal of IPTG.

While the toggle switch constructs of the invention are illustrated by the exemplary P_L-*lacI-Clone* and P_{trc}-*cI* operons in the toggle switch construct, the invention is not limited to the type of promoter, type of repressor protein or type of inducer used. Any repressor protein may be used so long as it reduces transcription by the promoter. Similarly, any inducer may be used so long as it increases transcription by the promoter. Prokaryotic and eukaryotic repressor-promoter-inducer combinations which are suitable for use in the toggle switch constructs are known in the art, such as those described in the Swiss-Prot protein database [Annotated Protein Sequence Database; http://expasy.hcuge.ch/sprot/sprot-top.html]. Suitable eukaryotic promoters are exemplified by the Human Cytomegalovirus Immediate Early Promoter (P_{hCMV}) [Gossen & Bujard (1992); Gossen, *et al.* (1995), *supra*], the Herpes Simplex Virus Thymidine Kinase Promoter (P_{HSVtk}) [Smith, *et al.* (1988), *supra*], and the Simian Virus Early Promoter [Wildeman, AG. (1988), *supra*]. Suitable prokaryotic promoters are exemplified by those in Table 1.

Table 1

Examples of *E. coli* constitutive promoters, repressors, and inducers suitable for toggle switch constructs, adjustable-threshold constructs and multi-state oscillator constructs

REPRESSOR	PROMOTER	INDUCER
ArsR	Arsenic operon	Arsenate or oxidized arsenic, antimony & bismuth
AscG	ASC operon[1]	*unknown*
LacI	P_{trc}	IPTG
CscR	Sucrose operon	D-fructose
DeoR (NucR)	Deoxyribose operon	deoxyribose-5-phosphate
DgoR	DGORKAT operon	D-galactonate
FruR	Fructose operon	D-fructose
GalR	Galactose operon	galactose
GatR	Galactitol operon[1]	*unknown*
CI	P_L	Nalidixic acid; UV light
LexA	SOS response regulon	UV light & RecA protein
RafR	Raffinose operon	raffinose
TetR	Tetracycline resistance operon	tetracycline

[1] Until the inducer is found for the ASC operon and Galactitol operon promoters, these promoters are suitable only for use in the adjustable-threshold switch and the multi-state oscillator.

Application of the inducers of Table 1 to to a cell which contains a repressor of Table 1 and its cognate constitutive promoter in the toggle switch construct results in switching expression of the *Clone* of Figures 1 and 20 from "on" to "off," or from "off" to "on."

Similarly, the invention is not limited to the relative locations of P_1, *Gene 1* and *Clone* of Figures 1 and 20. The invention expressly contemplates both P_1-*Gene 1-Clone* operons as well as P_1-*Clone-Gene 1* operons so long as each of *Gene 1* and *Clone* are operably linked to P_1. Moreover, the invention also includes having one gene of interest (*e.g., Clone 1*) operably linked to P1-*Gene 1* as well as another gene of interest (*e.g., Clone 2)* operably linked to P2-*Gene 2*. Furthermore, the invention also encompasses having the first operon (*i.e.*, P_1-*Gene 1-Clone* of Figure 1) and second operon (*i.e.*, P_2-*Gene 2* of Figure 1) on the same or on different vectors.

The toggle switch constructs of the invention may be used in clinical applications such as gene therapy. For example, recent work by Rendahl et al. [Rendahl, KG et al.(1998) Nature Biotechnology 16:757-761] demonstrated a successful method for the delivery and controllable expression of a recombinant *epo* gene in mice. This work demonstrates the feasibility of regulating expression of the *epo* gene (which stimulates the production of red blood cells) in the treatment of hemoglobinopathies or anemia in humans. In the Rendahl et al. scheme, the *epo* gene is placed under the control of a tetracycline-controlled transcriptional activator. The presence of tetracycline interferes with gene expression by binding to the transcriptional activator. Thus, the expression of the *epo* gene and the consequent production of red blood cells can be turned on and off by the administration of tetracycline.

While this approach seems promising, it suffers from the drawback that it requires sustained ingestion of tetracycline in order to maintain the suppressed state of the *epo* gene. Long-term ingestion of tetracycline may not be practical for a variety of reasons, such as side effects of long-term antibiotic administration and inconvenience. On the other hand, expression of *epo* gene (or any other transgene) under the control of the toggle switch constructs provided herein allows maintenance of gene expression in either the "on" or "off" state until the toggle is switched by the transient (rather than sustained) ingestion of the appropriate drug (*e.g.*, tetracycline).

Toggle switch constructs may also be used to control the cell cycle. For example, recent work has shown that a protein which reversibly binds any one of the cell-division cycle (CDC) proteins can modulate the frequency of cell division or stop and restart cell division completely [Gardner, TS. et al.(1998) Proc. Natl. Acad. Sci USA, 95: 14190-14195]. This scheme requires the controllable expression *in vivo* of the binding protein. The toggle switch construct is an ideal system for controlling expression of the binding

protein. It can be flipped "on" by transient administration of an inducer of one of the promoters in the toggle's two operons, thus causing the cell cycle to stop or its frequency to change. The cell will remain in this state until it is desired to restart the cell cycle or return it to its normal frequency. At such time, the toggle switch construct can simply be flipped again by transient application of an inducer of the other promoter in the toggle. Control of cell division in this manner may, for example, be applied to control cell growth, improve the manufacture of engineered tissues, and to treat cancer.

In addition to the above, the toggle switch constructs provided herein form the building block for the adjustable-threshold switch construct described below.

2. Adjustable-threshold Switches

The adjustable-threshold switch constructs provided herein are exemplified in Figure 5. Transcription of *Gene 1* by promoter 1, P_1, is activated by an activating agent or agents (*e.g.*, UV light, a chemical, a protein, temperature shift, *etc.*) exemplified by protein X, and simultaneously inhibited by the repressor protein encoded by *Gene 2*. Promoter 2, P_2, efficiently transcribes *Gene 2* unless inhibited by the repressor protein encoded by *Gene 1*. Open arrows indicate direction of transcription. *Clone* is an additional gene or genes which may be placed under the control of P_1 or P_2. One of skill in the art appreciates that in addition to the first *Clone* which is under the control of P_1, a second *Clone* (which is the same or different from the first *Clone*) may be placed under the control of P_2.

The arrangement of the adjustable-threshold switch construct is similar to that of the toggle switch construct with the exception that the first promoter is not constitutive. Transcription from the first promoter requires an activating agent or agents (*e.g.*, UV light, a chemical, a protein, temperature shift, *etc.*). In addition, the first promoter is repressed by the second protein. Furthermore, unlike the toggle switch construct in which transcription from either promoter is equally stable, the adjustable-threshold switch construct has a "default" transcription state in which transcription of genes from the first promoter is (for example) "off" while that from the second promoter is "on." The default transcription of the gene of interest may be manipulated such that it is in a default "off" or "on" state by inserting the gene of interest in downstream of either the first or second promoters, respectively. This system is able to sharply switch from transcription state 1 (*i.e.*, suppression of the first gene and transcription of the second gene) to transcription state 2 (*i.e.*, transcription of the first gene and suppression of the second gene) by the application of

the activating agent at a concentration that exceeds a desired threshold concentration. This sharp switching is exemplified by the toggle switch described herein (Example 2; Figure 25). The term "threshold concentration" as used herein in reference to an agent that is applied to a construct in which a first gene is under the control of a first promoter, and a second gene is under the control of a second promoter, wherein the first gene is not transcribed and the second gene is transcribed, refers to a concentration of the agent which is sufficient to bring about transcription of the first gene and to result in inhibition of transcription of the second gene. Preferably, the switching of transcription of the first gene from "off" to "on" in response to the threshold concentration of the agent is sharp, *i.e.*, application of the agent results in a relatively high level of transcription (as compared to the level of transcription in the absence of the agent) of the first gene.

A genetic switch with an adjustable switching threshold is produced by modification of the toggle switch design. Like the toggle, this device is composed of two mutually inhibitory genes. However, promoter 1 is modified such that it cannot transcribe *Gene 1* without aid of an additional activator agent. In the absence of the activator agent (*e.g.*, protein X), promoter 2 will dominate promoter 1 and *Gene 2* will be expressed. As the concentration of protein X rises, the strength of promoter 1 will rise as well. Eventually, the strength of promoter 1 will exceed that of promoter 2 and the device will abruptly switch to the expression of *Gene 1*. By manipulating the relative strengths (as described below) of promoter 1 (when activated) and promoter 2, the concentration of the activator agent at which this transition occurs can be altered. The adjustable-threshold switch construct is modeled by the following two Equations 3:

$$\frac{du}{dt} = \frac{k_1\lambda_1/\delta_1 x^\eta}{x^\eta + K_{mx}^\eta(1 + v^\gamma/K_{iv}^\gamma)} - d_1 u$$

$$\frac{dv}{dt} = \frac{k_2\lambda_2/\delta_2}{1 + K_{mv}(1 + u^\beta/K_{iu}^\beta)} - d_1 v$$

where,

x = concentration of transcriptional activator of promoter 1,

K_{mx} = Michaelis constant for activator binding and transcription of *Gene 1*,

η = cooperativity of binding of transcriptional activator.

All other parameters are as given in Equations 1 above. The three new parameters describe the activation of transcription by protein X. It is assumed that the activator, like the inhibitor proteins, can bind the DNA cooperatively and the activation can be modeled with Michaelis-Menton kinetics. In this model, protein X can be considered as the "input" to the switch. Increasing the concentration of protein X will cause the "output"' of the switch to flip from *Gene 2* expression to *Gene 1* expression.

Figure 6 provides an understanding of the switching mechanism. Bold line is nullcline for dv/dt = 0. Light lines are nullclines for du/dt = 0. For x=0.5, state 1 is the only stable fixed point. As x increases, the system remains in state 1 until x is approximately 2. A bifurcation eliminates state 1 and the system switches dramatically to state 2.

The initial state of the system, state 1 (*i.e.*, no activator protein, expression of *Gene 2*, suppression of *Gene 1*), is the stable fixed point which occurs at the intersection of nullcline 2a with nullcline 1. When the concentration of protein X rises, the shape of nullcline 2a is altered to nullcline 2b, causing it to intersect with nullcline 1 in two additional places. In this intermediate state, the system exhibits bi-stability analogous to that of the toggle switch construct. However, the system will remain in state 1 in the absence of a large perturbation. As described below, this bistability leads to hysteresis in the switching mechanism. Depending on the intended application, hysteresis may or may not be useful. Tuning the system (*e.g.*, by manipulating the strengths of promoter 1 (α_1) and of promoter 2 (α_2) as described below) allows the size of this hysteresis to be adjusted. When the concentration of protein X is further increased, the nullcline shifts to curve 2c, the stable fixed point at state 1 disappears and state 2 (expression of *Gene 1*, suppression of *Gene 2*) becomes the sole stable fixed point. Thus, the system dramatically shifts from state 1 to state 2. This shift, which was driven by the change in protein X concentration, is the threshold of the system. It can be altered by adjusting the nullclines whose shape and location are determined by the parameters in Equations 3.

As with the toggle, the analysis of this system is simplified by rescaling time and non-dimentionalizing the variables. Equations 1 are thus reduced to the following Equations 4:

$$\frac{d\hat{u}}{d\tau} = \frac{\alpha_1 \hat{x}^{\eta}}{\hat{x}^{\eta} + 1 + \hat{v}^{\beta}} - \hat{u}$$

$$\frac{d\hat{v}}{d\tau} = \frac{\alpha_2}{1 + u^{\gamma}} - \hat{v} \qquad \textit{where,}$$

$$\tau = d_1 t,$$

$$\hat{u} = \frac{u}{K_{iu}(1 / K_{mv} + 1)^{1/\beta}},$$

$$\hat{v} = \frac{v}{K_{iv}},$$

$$\hat{x} = \frac{x}{K_{mx}},$$

$$\alpha_1 = \frac{k_1 \lambda_1 / \delta_1}{d_1 K_{iu} (1 / K_{mv} + 1)^{1/\beta}} \qquad \textit{and,}$$

$$\alpha_2 = \frac{k_2 \lambda_2 / \delta_2}{d_1 K_{iv} (1 + K_{mv})}.$$

Once again, nine parameters in the original equations have been collapsed into two dimensionless parameters. Thus, analysis of the effects of only five parameters (*i.e.*, x, $\alpha 1$, $\alpha 2$, η, and γ) is required.

Figure 7 shows the structure of the threshold. Hysteresis exists at the switching threshold as indicated by the arrows in plots A and B. Both the location of the threshold and size of the hysteresis can be manipulated independently. Parameter values: α_1=5, α_2=14 (curve a, both plots), α_1=3, α_2=10 (curve b, both plots), α_1=2.7, α_2=15 (curve c, both plots), $\eta=\gamma=2$ (all curves, both plots). Figure 7 shows the steady-state concentrations of proteins u and v versus the concentration of x for several values of parameters α_1 and α_2. This figure reveals more clearly the nature of the threshold and the associated hysteresis. It also demonstrates that both the location of the threshold and the size of the hysteresis can be tuned.

The effects of parameters α_1 and α_2 on the threshold and hysteresis are more fully illustrated in Figure 8. Figure 8 is a bifurcation diagram showing the size of the hysteresis and the location of the threshold for a range of values of promoter strengths in an adjustable-threshold switch construct. In both plots, the lines demarcating the hysteresis region are saddle-node bifurcations. To achieve minimal hysteresis, a weak promoter 1 is desirable; however, a minimal promoter strength exists beyond which no threshold will be generated. This minimal strength can be determined through experimental adjustment and testing of promoter strengths. Increasing the strength of promoter 2, or decreasing the strength of promoter 1, translates the threshold to higher values of x. Parameter values: $\eta = \gamma = 2$ for all curves; (A) $\alpha_2=2$ (curve a), $\alpha_2=10$ (curve b), $\alpha_2=20$ (curve c); (B) $\alpha_1=10$ (curve a), $\alpha_1=5$ (curve b), $\alpha_1=3$ (curve c). Increases in α_2 (*i.e.*, the strength of promoter 2) move the switching threshold to larger concentrations of protein X, but also increase the size of the hysteresis. Reductions in α_1, the maximum strength of promoter 1, also move the threshold to higher concentrations of protein X and simultaneously reduce the size of the hysteresis. Thus, a switch with the desired threshold and hysteresis characteristics can be designed by choosing promoters of appropriate strengths (*i.e.*, those strengths which produce the desired threshold and hysteresis characteristics. Strengths would be adjusted according to the qualitative predictions of the theory. Manipulations and assays are the same as those described for the toggle switch construct). Finally, Figure 8a shows that there are absolute limits to the adjustability of the threshold. For very low values of α_1, no switching occurs regardless of the strength of promoter 2. Furthermore, in the experimental system, the promoter strength will have a physically determined maximum that will place an upper limit on the location of the switching threshold.

The above discussion shows that, based on the inventors' preliminary determination, the adjustable-threshold construct's characterizing sharp rise in transcription of the gene of interest requires that (a) where the first and second repressor proteins form homo-dimers, both α_1 and α_2 have a value of greater than 2, and (b) where the first and second repressor proteins form homo-multimer other than dimers, either α_1 or α_2 must have a value greater than 1. Like the toggle switch construct, the minimum permissible value of α_1 or of α_2 approaches 1 as the degree of multimerization increases, but it never falls below 1.

The toggle and threshold constructs are distinguished from each other in the function of the agent used to bring about switching from one transcription state to another. Specifically, while the agent in the toggle switch construct inhibits the activity of the

repressor proteins (*i.e.*, either repressor protein 1 or 2), the agent in the threshold switch construct activates the first promoter. The threshold concentration of the activating agent (*e.g.*, activator protein X) at which the switch from transcription state 1 to state 2 is achieved may be manipulated by adjusting the strengths of the first and second promoters as described below.

The repressors, activators and promoters used in the adjustable-threshold switch constructs of the invention are not intended to be limited to any particular type or source. Any combination of inducible promoters (and their cognate activators) and constitutive promoters (and their cognate repressors) is suitable for use in the adjustable-threshold switch construct. Suitable promoters and cognate repressors/activators are known in the art (*e.g.*, those contained in the Swiss-Prot protein database at http://exasy.hcuge.ch/sprot/sprot-top.html) and include those listed in Tables 1 and 2 and the eukaryotic promoters P_{hCMV}, P_{HSVtk}, P_{SV40}. In addition, artificial eukaryotic activators can be constructed from DNA binding proteins fused with the activation domains such as the Herpes Simplex Virus VP16 activation domain [Gossen & Bujard (1992), *supra*], the human B42 activation domain [Clontech Laboratories, http://www.clontech.com], or the yeast GAL4 activation domain [Darnell, *et al.* (1990), *supra*]. The cognate inducible promoter is constructed from the DNA recognition sequence of the binding domain fused with a portion of a constitutive eukaryotic promoter.

The inducible promoters (*i.e.*, P_1 in Figure 5) of the adjustable-threshold switch constructs provided herein preferably direct only low levels of expression of genes under their control in the absence of the activating agent. The term "low level of expression" when made in reference to a gene means that the quantity of protein expressed by the gene is preferably below the threshold concentration (Figures 7 and 8) as detected by, for example, an Enzyme Linked Immunosorbent Assay (ELISA). When a background level or undetectable level of the protein is measured, this may indicate that the protein is not expressed. In addition, the adjustable-threshold switch constructs require an inducible promoter that is capable of both being activated by an activating agent (*e.g.*, Protein X in Figure 5) and of being suppressed by a protein (*i.e.*, the expression product of *Gene 2* of Figure 5). Prokaryotic and eukaryotic promoters which satisfy these criteria are known in the art (*e.g.*, those contained in the Swiss-Prot protein database at http://exasy.hcuge.ch/sprot/sprot-top.html), and can be constructed from combinations of inducible and repressible promoters. Examples of repressible prokaryotic promoters are

listed in Table 1, *supra*, and examples of inducible prokaryotic promoters are listed in Table 2.

Table 2

Examples of *E. coli* inducible promoters, and activators suitable for adjustable-threshold constructs and multi-state oscillator constructs

Activator	Promoter[1]	Co-activator
AraC	Arabanose operon	arabanose
CadC	P_{cad} (CAD Operon)	low pH
CRP	*deoP2*	cAMP
CynR	Cyn operon	cyanate
DsdC	Dsd operon	CRP, cAMP
FhlA	Formate dehydrogenase/hydrogenase genes	formate
MalT	*malPp*	maltose
MaoB	Monoamine oxidase gene	CRP, cAMP, tyramine
IlvY	IlvC gene	acetolactate, acetohydroxybutyrate
UreR	Urease operon	urea

[1] Transcription from the promoters is induced in the presence of both activator and co-activator.

Furthermore, the adjustable-threshold switch constructs provided herein are not intended to be limited to the location of *Gene 1* and *Clone* of Figure 5 in relation to the first promoter P_1. Rather, the adjustable-threshold switch constructs of the invention are expressly contemplated to encompass both P_1-*Gene 1*-*Clone* operons as well as P_1-*Clone*-*Gene 1* operons so long as each of *Gene 1* and *Clone* are operably linked to P_1. Moreover,

the invention also includes having one gene of interest (*e.g., Clone 1*) operably linked to P1-*Gene 1* as well as another gene of interest (*e.g., Clone 2)* operably linked to P2-*Gene 2*. In addition, the invention also encompasses having the first operon (*i.e.*, P_1-*Gene 1-Clone* of Figure 5) and second operon (*i.e.*, P_2-*Gene 2* of Figure 5) on the same or on different vectors.

The invention's adjustable-threshold switch constructs have clinical applications such as in gene therapy. For example, the adjustable-threshold switch construct would be constructed such that it activates or inactivates the expression of a desired transgene in response to changes in the concentration of a particular compound in the body. Currently, sufferers of diabetes must inject insulin into their blood-stream when their blood glucose is abnormally elevated. In a gene therapy treatment of this disease, liver cells are transfected with an insulin gene that is under the control of the adjustable-threshold switch construct. When blood glucose levels are elevated above a predetermined threshold level, the switch would initiate production of insulin. Thus, the individual with diabetes would be freed of the need for constant insulin injections.

The adjustable-threshold switch constructs may also be used as chemical and/or protein sensors and switches. In investigating gene expression, it may be desirable to monitor *in vivo* or *in situ* the concentration of certain chemical compounds or proteins. The adjustable-threshold switch constructs would be designed to express the Green Fluorescent Protein (Clontech) when the concentration of the protein or compound of interest rises above or falls below a particular threshold levels of the chemical compound or protein of interest. A highly sensitive version of this system could be used as the basis of an *in vivo* system for the detection of biological or chemical warfare agents.

Moreover, the adjustable-threshold switch constructs may also be used to activate other genes in response to changes in the cellular concentration of a particular protein or compound. For example, the switch construct could be designed to activate a gene once per cell division by linking its expression to the concentration of metaphase promoting factor.

In addition to the above uses, the adjustable-threshold switch constructs provided herein form the central component of the multi-state oscillator constructs described below.

3. Multi-state Oscillators

Multi-state oscillators are exemplified by the two-state oscillator construct. The two-state oscillator construct may be arranged in three alternative configurations. In each configuration, oscillatory behavior is achieved by adding to the adjustable-threshold switch construct a feedback loop comprised of one or two additional genes and one additional promoter.

The first configuration of the two-state oscillator construct is illustrated in Figure 9A. In this configuration, a third regulatory gene (*Gene 3*) is placed under the control of the second promoter, P_2, in tandem with the second gene of the adjustable-threshold construct. A fourth gene (*Gene X*) is placed under the control of a third promoter (P_3). The product of the third gene activates the expression of a fourth gene from the third promoter. The product of the fourth gene (*Gene X*) activates expression from the first promoter.

In the second configuration of the two-state oscillator construct (Figure 9B), the third gene (*Gene 3*) is placed under the control of the first promoter. The fourth gene (*Gene X*) and the third promoter are arranged as in the first configuration; however, the product of the third gene inhibits expression from the third promoter.

In the third configuration of the two-state oscillator construct (Figure 9C), the product of *Gene 1* inhibits expression from both the second (P_2) and third promoters (P_3), the product of *Gene 2* inhibits expression from the first promoter (P_1), while the product of *Gene X* activates epxression from the first promoter (P_1).

One of skill in the art appreciates that in each of the three configurations of the two-state oscillator construct, in addition to the first *Clone* which is under the control of P_1, a second *Clone* (which is the same or different from the first *Clone*) may be placed under the control of P_2.

The two-state oscillator construct is characterized by being able to generate sustained periodic expression of the genes in the first operon (*i.e.*, P_1-*Gene 1-Clone* of Figure 9A, and P_1-*Gene 3-Gene 1-Clone* of Figure 9B) and genes in the second operon (*i.e.*, P_2-*Gene 2-Gene 3* of Figure 9A, and P_2-*Gene 2* of Figure 9B) without the addition of a stimulus which

is extraneous to the products of the three operons. This behavior is produced by adjusting the strengths of the promoters and the stability of the protein products as described below. In addition, the period of oscillations may also be manipulated.

The oscillator, in the first configuration, is modeled by adding a delay equation to the threshold equations (Equations 4). The inventors' preliminary determination yielded the following Equations 5.

Equations 5:

$$\frac{d\hat{u}}{d\tau} = \frac{\alpha_1 \hat{x}^{\eta}}{\hat{x}^{\eta} + 1 + \hat{v}^{\beta}} - \hat{u}$$
$$\frac{d\hat{v}}{d\tau} = \frac{\alpha_2}{1 + u^{\gamma}} - \hat{v}$$
$$\frac{d\hat{x}}{d\tau} = \frac{K_d \hat{v} - \hat{x}}{\kappa_d}$$

In a more preferred embodiment, the inventors' determination yielded the following Equations 6:

$$\frac{d\hat{u}}{d\tau} = \frac{\alpha_1 \hat{x}^{\eta}}{\hat{x}^{\eta} + 1 + \hat{v}^{\beta}} - \hat{u}$$
$$\frac{d\hat{v}}{d\tau} = \frac{\alpha_2}{1 + u^{\gamma}} - \hat{v}$$
$$\frac{dx}{dt} = \frac{1}{\kappa_d}\left(\frac{K_d v^{\rho}}{1+v^{\rho}} - x\right)$$

where,

K_d = strength of promoter 3,

κ_d = time delay which is the time it takes for x to reach 2/3 of its steady-state concentration for a given value of v.

ρ = cooperativity of binding of third protein to third promoter.

The other parameters in Equations 5 and 6 are the same as given in Equations 4.

The results of simulations of Equations 5 are shown in Figure 10A and 10B and of Equations 6 are shown in Figure 10C and 10D. Parameter values: α_1=α_2=10, κ_d = 0.6. Figure 10 shows the predicted behavior of the system. By adjusting the time delay, the period of oscillations can be altered. (Compare Figures 10A and 10B). The time delay

describes the lag in the response of the protein X relative to the proteins U and V. Thus, the time delay can be increased, for example, either by decreasing the rate of degradation of the protein X or by increasing the rate of degradation of the proteins U and V. Furthermore, the proper tuning of this time delay and the strength of promoter 3 is critical for the production of oscillations. If the delay is too long or too short, or if the promoter is too weak or too strong, the system will not oscillate; it will settle to a steady-state. Both of these parameters can be manipulated experimentally using methods known in the art to achieve the desired behavior. The time-delay and promoter strength may be adjusted according to the qualitative predictions of the model. That is, based on experimental measurements of the system's behavior, the promoter strength and time delay may be increased or decreased until oscillations are experimentally observed (*e.g.*, through the use of a reporter gene). The strength and time-delay may be altered by manipulating the DNA sequence of the promoters and the genes. The relative strengths of the promoters that compose the adjustable-threshold switch portions of the oscillator are as described for the adjustable threshold switch.

It is not intended that the repressors, activators and promoters used in the two-state oscillator constructs of the invention be limited to a particular type or source. Any combination of inducible promoters (and their cognate activators) and constitutive promoters (and their cognate repressors) is suitable for use in the two-state oscillator constructs. Suitable promoters and cognate repressors/activators are known in the art (*e.g.*, those contained in the Swiss-Prot protein database at http://exasy.hcuge.ch/sprot/sprot-top.html) and include those listed in Tables 1 and 2 and the eukaryotic promoters P_{hCMV}, P_{HSVtk}, P_{SV40}. In addition, artificial eukaryotic activators can be constructed from DNA binding proteins fused with the activation domains such as the Herpes Simplex Virus VP16 activation domain [Gossen & Bujard (1992), *supra*], the human B42 activation domain [Clontech Laboratories, http://www.clontech.com], or the yeast GAL4 activation domain [Darnell, *et al.* (1990), *supra*]. The cognate inducible promoter is constructed from the DNA recognition sequence of the binding domain fused with a portion of a constitutive eukaryotic promoter.

Furthermore, the two-state oscillator constructs provided herein are not intended to be limited to the location of *Gene 1* and *Clone* in relation to P_1 of Figure 9A, *Gene 2* and *Gene 3* in relation to P_2 of Figure 9A, of *Gene 1*, or of *Gene 3* and *Clone* in relation to P_1 of Figure 9B. Moreover, the invention also includes having one gene of interest (*e.g.*,

Clone 1) operably linked to P1-*Gene 1* as well as another gene of interest (*e.g., Clone 2)* operably linked to P2-*Gene 2-Gene 3* of Figure 9A. The invention also expressly contemplates having one gene of interest (*e.g., Clone 1*) operably linked to P1-*Gene 3-Gene 1* as well as another gene of interest (*e.g., Clone 2)* operably linked to P2-*Gene 2* of Figure 9B. One of skill in the art knows that various configurations of the genes and promoters may be used to achieve the desired behavior of the two-state oscillator constructs so long as genes are operably linked to the promoters. Furthermore, it is expressly contemplated that each of the operons of Figure 9 may be on the same or on different vectors.

The invention's two-state oscillator constructs have clinical applications such as in gene therapy. It is known that the concentration of certain hormones in the body fluctuates periodically during the day. Currently, treatment of diseases in which these hormones are deficient may be accomplished through the periodic ingestion of drugs in order to bring about a periodic change in the concentration of the deficient hormone. Such treatments may be achieved instead by placing the missing or damaged gene which encodes the hormone in question under the control of a two-state oscillator of the invention. The period of the oscillator could be adjusted appropriately such that the hormone is periodically expressed without the need for drug ingestion and such that the periodic change in the concentration of the expressed hormone mimics that in the normal state.

The two-state oscillator constructs may also be used to control the cell cycle. For example, two-state oscillator constructs may be used to actively alter the CDC frequency. In active control, a construct with inherent oscillations could be coupled to the cell cycle and drive it at a new frequency. Such a function could be carried out by a two-state oscillator which periodically expresses one of the CDC proteins or a binding protein. Furthermore, the frequency of the two-state oscillator, and hence, the frequency of cell division, could be dynamically controlled by modulating, with external chemical signals, the time-delay in the feedback loop.

B. Construction of Genetic Applets

The following describes the various ways in which each of the toggle switch constructs, adjustable-threshold switch constructs and multi-state oscillator constructs may be manipulated to achieve the desired behaviors described *supra*.

1. Experimental Manipulation of System Parameters

Promoter strength in each of the toggle switch constructs, adjustable-threshold switch constructs and multi-state oscillator constructs may be altered in prokaryotic and eukaryotic cells by manipulating one or more of the following: the strength of RNAP binding to DNA (K_{mu} or K_{mv}), the maximum rate of mRNA synthesis by RNAP $\ddot{e}_1$ or $\ddot{e}_2$), the strength of inhibitor binding to the DNA (K_{iu} or K_{iv}), the strength of activator binding to DNA (K_{mx}), the rate of translation of mRNA into functional protein (k_1 or k_2), and the rate of protein degradation, i.e., protein stability (d_1). These are further described below.

i. RNAP Binding

In prokaryotic cells, recognition of the promoter sequence by RNAP is mediated by helper proteins called sigma factors that bind to two sites in the promoter: the Pribnow box (or -10 region) and the -35 region. Each of these sites has an ideal sequence called a consensus sequence. The strength of binding of sigma factors, and thus the strength of RNAP binding, is determined by how closely these regions match their consensus sequence [Darnell et al (1990), *supra*]. Furthermore, modifications of a region upstream of the -35 region, called the UP element, have been shown to dramatically alter the rate of transcription [Estreem, ST et al. (1998) Proc. Natl. Acad. Sci. USA 95:9761-9766; Yamada, M, et al. (1991) Gene 99:109-114]. The UP element, which has also been shown to have a consensus sequence, probably enhances the binding of the RNAP complex. By modifying the sequence of the -10, -35 and UP regions, *e.g.*, by introducing a deletion, point mutation or insertion, the strength of RNAP binding and, hence, the promoter strength, can be altered. Relative promoter strengths can be determined be quantitative assays of the expression of reporter genes such as the green fluorescent protein (GFP), β-galactosidase (β-gal), or chloramphenicol acetyl transferase (CAT). Thus one of skill in the art may determine whether, for example, a mutation has increased or decreased the level of expression of a gene.

ii. Transcription Elongation

Once the RNAP binds to a promoter, it opens the DNA double helix and moves forward, adding ribonucleotides to the mRNA transcript. The rate of transcription is determined partially by the nucleotide content and partially by the secondary structure (if any) of the mRNA. High guanosine and cytosine content will slow the transcription rate

[Darnell et al. (1990), *supra*]. Secondary structures that form in the mRNA behind the transcription complex can interfere with the transcription process [Darnell et al. (1990), *supra*]. Although the DNA content of the coding region cannot be substantially altered (only silent mutations will alter the mRNA sequence without changing the protein properties), a leader region of mRNA may be inserted upstream of the coding region. This region can be designed to slow the rate of transcription elongation. A change in the rate of transcription elongation may be determined using methods known in the art. For example, pulse labeling mRNA transcripts with radioactive nucleotides can be used to track mRNA both temporally and spatially.

iii. Inhibitor-Activator Binding

Special sequences of DNA called operators are often found within or near a promoter. The inhibitor proteins (repressors) block transcription by binding to these operators. On the other hand, when an activator binds to an operator, it increases the binding affinity and/or transcription rate of the RNAP. A given repressor or activator will recognize only a specific operator sequence. The affinity of the repressor/activator for the operator can be altered by modifying the operator sequence, *e.g.*, by introducing a point mutation, insertion or deletion.

iv. Translation Rate

The rate of translation of mRNA into an amino-acid sequence is governed primarily by three factors: the ribosome binding site (RBS), the secondary structure of the mRNA, and the codon content of the coding region. The RBS is located 5-10 bases upstream of the start codon. Translation is most efficient when this sequence matches a consensus sequence called the Shine-Dalgarno (SD) sequence [Darnell et al. (1990), *supra*, Backman, K & Ptashne, M. (1978) Cell 13:65-71; Jacques, N & Dreyfus, M. (1990) Molecular Microbiology 4:1063-1067; Shine, J & Dalgarno, L. (1975) Nature 254:34-38]. Thus, translation rate can be altered by modifying the RBS, *e.g.*, by introducing a point mutation, insertion or deletion. As in transcription, the formation of secondary structures by the mRNA can interfere with translation machinery. Thus, modification of the leader region of the mRNA or introduction of silent mutations into the coding region may be used to change translation rate. Finally, in various organisms certain codons are favored, *i.e.*, tRNAs for certain codons are more abundant than others. Translation is more efficient when the

favored codons are used [Jacques & Dreyfus (1990), *supra*]. Thus, a coding region can be optimized by introducing silent mutations that utilize the favored codons.

v. Protein Stability

The stability of a protein can be altered by introducing mutations into the amino acid sequence that make the protein more or less resistant to denaturation or proteolytic degradation. Powerful experimental techniques such as directed evolution, DNA shuffling and two-hybrid screening are known in the art and may be used to rapidly screen large numbers of mutant proteins for the desired stability characteristics. In addition, protein degradation rate may be altered by attaching a short, organism-specific, oligonucleotide sequence [Andersen *et al.* (1998) Appl. Environ. Microbiol. 64:2240-2246] to the 3' end of the gene which encodes the protein. This sequence targets the encoded protein for rapid degradation by the cell.

2. Construction

i. Organisms

The methods of the invention are not intended to be limited to the type of organism into which the constructs of the invention are to be introduced. Rather, any organism is contemplated to be within the scope of the invention. Such organisms include, but are not restricted to, non-human animals (*e.g.*, vertebrates, invertebrates, mammals, fish, insects, *etc.*), plants (*e.g.*, monocotyledon, dicotyledon, vascular, non-vascular, seedless, seed plants, *etc.*), protists (*e.g.*, algae, citliates, diatoms, *etc.*), fungi (including multicellular forms and the single-celled yeasts), bacteria (prokaryotic, eukaryotic, archaebacteria, *etc.*), and viruses.

In one preferred embodiment, the organism is a prokaryote. In another preferred embodiment, the organism is a eukaryote.

ii. Cells

Any type of cell capable of being transfected with a the constructs of the invention (*i.e.*, toggle switch construct, adjustable-threshold construct, and multi-state oscillator construct) is expressly included within the scope of this invention. Such cells are preferably, though not necessarily, capable of being cultured. The term "cell capable of being cultured" as used herein refers to a cell which is able to divide *in vitro* and/or *in vivo* to produce two or more progeny cells. Such cells are exemplified by embryonic cells (*e.g.*,

embryonic stem cells, fertilized egg cells, 2-cell embryos, protocorm-like body cells, callus cells, *etc.*), adult cells (*e.g.*, brain cells, fruit cells, *etc.*), undifferentiated cells (*e.g.*, fetal cells, tumor cells, *etc.*), differentiated cells (*e.g.*, skin cells, liver cell, *etc.*), animal cells, plant cells, prokaryotic cells, eukaryotic cells, and the like. In a preferred embodiment, the cell is a prokaryotic cell. In another preferred embodiment, the cell is a eukaryotic cell.

iii. Modification of Nucleic Acid Sequences

Construction of the genetic applets of the invention contemplates modification of nucleic acid sequences (*e.g.*, promoters) in order to attain certain desired properties. These properties include, but are not limited to, the concentration of the switching threshold, the time necessary to complete switching, the level of expression of the gene of interest in the "on" or "off" states, the size and position of the hysteresis, the relative stability of the stable states, the frequency of oscillations and the amplitude of oscillations. A "modification" as used herein in reference to a nucleic acid sequence refers to any change in the structure of the nucleic acid sequence. Changes in the structure of a nucleic acid sequence include changes in the covalent and non-covalent bonds in the nucleic acid sequence. Illustrative of these changes are point mutations, mismatches, and strand breaks. Point mutations include deletions, insertion and substitutions.

Nucleic acid sequences may be modified using methods known in the art including, but not limited to, the use of chemicals, use of electromagnetic radiation, site-directed mutagenesis, *etc.* Exemplary chemicals are described at http://dir.niehs.nih.gov/dirtb/dirrtg/chemicalsstudiedindex2.htm including (*e.g.*, *N*-ethyl-*N*-nitrosurea (ENU), methylnitrosourea (MNU), procarbazine hydrochloride (PRC), triethylene melamine (TEM), acrylamide monomer (AA), chlorambucil (CHL), melphalan (MLP), cyclophosphamide (CPP), diethyl sulfate (DES), ethyl methane sulfonate (EMS), methyl methanes ulfonate (MMS), 6-mercaptopurine (6MP), mitomycin-C (MMC), procarbazine (PRC), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), ^3H_2O, and urethane (UR). Electromagnetic radiation is exemplified by ultraviolet light, X-ray radiation, gamma-radiation, *etc.*

iv. Introduction of Vectors Into Host Cells

Once a genetic applet is constructed, it may be inserted into a vector. As used herein, the terms "vector" and "vehicle" are used interchangeably in reference to nucleic acid molecules that transfer DNA sequences from one cell to another, and are exemplified by plasmids, recombinant bacteriophage, cosmid DNA vectors, viruses and the like. In one preferred embodiment, the vector is a plasmid.

Vectors may be introduced into a host cell using a number of standard and routine methods known to those skilled in the art [see, *e.g.*, Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, N.Y. Cold Spring Harbor Laboratory (1989)] including, but not limited to, microinjection, DEAE-dextran, calcium phosphate co-precipitation, cell fusion, electroporation, biolistics, lipofection, DNA viruses, and RNA viruses, or retrovirus-mediated transduction.

Where the host cell is a plant cell, vectors may be introduced by particle mediated gene transfer. Particle mediated gene transfer methods are known in the art, are commercially available, and include, but are not limited to, the gas driven gene delivery instrument descried in McCabe, U.S. Patent No. 5,584,807 (herein incorporated by reference), infecting plant cells with a bacterium (*e.g.*, *Agrobacterium tumefaciens* or Ri plasmids of *Agrobacterium rhizogenes* such as those described in U.S. Patent No. 4,940,838 (herein incorporated by reference), electroinjection, fusion with liposomes, lysosomes, cells, minicells or other fusible lipid-surfaced bodies, chemicals that increase free DNA uptake, transfectation using virus, and the like.

v. Design Considerations For Prokaryotic Cells

In one preferred embodiment, the cell containing the genetic applets of the invention is a prokaryotic cell. In a more preferred embodiment, the prokaryotic cell is *E. coli*. While the toggle switch construct is illustrated herein by the P_L-*lacI-GFPuv* and P_{trc}-*cI* operons which are inserted into the pTAK1 plasmid, those of ordinary skill in the art recognize that the modular design of pTAK1 allows insertion of any promoter and any coding sequence into this plasmid to obtain the configurations of toggle switch constructs, adjustable-threshold constructs, and multi-state constructs of the invention. For example, where it is desirable to insert a repressor gene other than *lacI* and/or *cI* in pTAK1, the repressor gene is amplified and inserted in place of *lacI* and/or *cI*. Repressor genes may be obtained from wild-type *E. coli* or as plasmids from a number of commercial suppliers.

The repressor gene sequence may be amplified using methods known in the art, such as polymerase chain reaction (PCR). The term "amplify" and its grammatical equivalents is defined as the production of additional copies of a nucleic acid sequence. As used herein, the term "polymerase chain reaction" ("PCR") refers to the method of K.B. Mullis disclosed in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,965,188, all of which are hereby incorporated by reference, which describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (*i.e.*, denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified."

While the invention is illustrated using *E. coli*, constructs containing toggle switches, adjustable-threshold switches, and multi-state oscillators which function in other prokaryotic cells are expressly contemplated to be within the scope of the invention. Switches which contain *E. coli* promoters may readily be modified to bring about transcription of *Clone* in each of Figures 1, 5, and 9. Such modification is illustrated by Figure 16.

In Figure 16, Promoter 1, P_1, and promoter 2, P_2, are strong constitutive promoters from the selected host organism (prokaryotic or eukaryotic). *Gene 1* encodes a bacterial repressor that binds to the O_1 operator site and represses transcription from P_2. *Gene 2* encodes a bacterial repressor that binds to the O_2 operator site and represses transcription from P_1. Open arrows indicate direction of transcription. *Clone* is an additional gene or

genes which may be placed under the control of P_1 or P_2. In this configuration, P_1 and P_2 are constitutively transcribed in the selected host organism, but they lack regulatory sequences needed to repress expression. These regulatory sequences are provided by the operator sites spliced within or downstream of the constitutive promoters. The operator sites, which are derived from any *E. coli* promoter or from a promoter in the host organism, bind their associated repressor protein encoded by *Gene 1* or *Gene 2*. Thus, two fusion promoters are created that are efficiently transcribed in the host organism and repressed by the selected *E. coli* repressors, or by a repressor chosen from the host organism. This same scheme can be applied to the adjustable-threshold switch construct and the two-state oscillator construct to produce constructs that are functional in any selected organism.

vi. Design Considerations For Eukaryotic Cells

Constitutive eukaryotic promoters are composed of two elements: the minimal promoter sequence, for example from base pairs +1 to -65, and an enhancer sequence encompassing several hundred base pairs upstream of the minimal promoter. The minimal promoter sequence contains the TATA box consensus sequence and is necessary but not sufficient for RNA polymerase II binding and transcription. In the absence of the enhancer the minimal promoter does not efficiently initiate transcription [Darnell, J., *et al.* (1990) *supra*, Gossen, M. & Bujard, J. (1992) *supra*; Lubon, H., *et al.* (1989) Molecular and Cell Biology, 9: 1342-1345; Thomsen, DR., *et al.* (1984) *supra*.] Thus, a strong eukaryotic constitutive promoter requires both a minimal promoter region and an upstream enhancer region. Exemplary strong constitutive eukaryotic promoters which direct efficient transcription in the absence of an activator and which lack an operator sequence are known in the art (*e.g.*, those disclosed in the Swiss-Prot protein database) and are exemplified by those listed in Table 3.

Table 3

Examples of Strong Constitutive Eukaryotic Promoters

Promoter	Parent Organism/Gene
P_{hCMV}	Human Cytomegalovirus Immediate Early Promoter [Gossen, M. & Bujard, H. (1992); Gossen, M., *et al.* (1995)]
P_{HSVtk}	Herpes Simplex Virus Thymidine Kinase Promoter [Smith, GM., *et al.* (1988) EMBO J., 7: 3975-3982]
P_{SV40}	Simian Virus Early Promoter [Wildeman, AG. (1988)]

While the promoters in Table 3 direct efficient transcription, these promoters are not repressed because they lack an operator sequence. Thus, in order to repress the exemplary promoters in Table 3, operator sequences need to be operably ligated to the promoter sequence.

In the absence of the enhancer, transcription may be efficiently induced by an activator protein that binds to a region upstream of the minimal promoter. Eukaryotic activator proteins typically consist of two functionally distinct and separable domains: the DNA binding domain (BD) which recognizes a specific sequence, and an acidic activation domain (AD) which stimulates transcription initiation. Any DNA binding protein may be fused to the AD to create an artificial activator protein [*Smith, GM. (1988) supra*]. As described below, this unifying feature of eukaryotic gene regulation facilitates the construction of eukaryotic versions of the toggle switches, adjustable-threshold switches and multi-state oscillator switches which are exemplified herein by the *E. coli* genetic switches.

Because of the differences between eukaryotic and prokaryotic transcriptional machinery, a bacterial promoter will not be recognized and transcribed by the eukaryotic RNA polymerase II. However, it has been previously shown that hybrid eukaryotic promoters (*i.e.*, promoters composed of a constitutively transcribed eukaryotic promoter and a bacterial operator sequence) both are efficiently transcribed in the absence of the associated bacterial repressor protein, and are effectively repressed in the presence of the bacterial repressor. For example, a hybrid promoter was constructed by splicing the *E. coli*

LexA operator sequence into the HSV *tk* promoter. Expression from this promoter was reduced 10-fold in mammalian cells that synthesized the *E. coli* LexA repressor protein [Smith, GM. (1988) *supra*]. Thus, toggle switches, adjustable-threshold switches, and multi-state oscillator switches which are functional in eukaryotic cells may be constructed using an approach similar to that described above for prokaryotic *E. coli*.

For example, a eukaryotic toggle switch can be constructed from hybrid promoters containing a constitutive eukaryotic promoter and an appropriate *E. coli* operator as shown in Figure 16. This is illustrated by the construction of the exemplary toggle switch containing the P_{hCMV} promoter, the *lex A* gene and the *lacI* gene as shown in Figure 17. The Human Cytomegalovirus Immediate Early Promoter, P_{hCMV} directs constitutive transcription of both genes *lexA* and *lacI*. The LexA protein, encoded by the *lexA* gene, represses transcription at operator site O_{lex}. The LacI protein, encoded by the *lacI* gene, represses transcription at operator site O_{lac}. *Clone* is an additional gene or genes which may be placed under the control of either P_{hCMV} promoter.

To construct a eukaryotic version of the adjustable-threshold switch and the two-state oscillator, inducible eukaryotic promoters are also needed. Construction of such inducible promoters is facilitated by the modular design of eukaryotic promoters. The enhancer domain from the first toggle switch promoter is replaced with a DNA sequence recognized by the BD of the desired activator protein. Any *E. coli* DNA binding protein such as those listed in Table 1 may be used as the BD of an activator protein. The DNA binding protein would simply be fused with an AD such as the HSV VP16 domain. Other suitable activation domains which require inclusion of a DNA binding domain in the same construct are known in the art (*e.g.*, those listed in the Swiss-Prot protein database) and are exemplified by those listed in Table 4.

Table 4

Examples of Constitutive Eukaryotic Promoters

Activation Domain	Parent Organism
VP16	Herpes Simplex Virus [Gossen & Bujard (1992)]
B42	Human [Clontech Laboratories, http://www.clontech.com]
GAL4	Yeast [Darnell, *et al.* (1990)]

Because the fusion protein containing the BD and the AD is an artificial construct, it must be included in the adjustable-threshold construct. This can be accomplished by, for example, operably linking the gene for the fusion protein to a constitutive promoter which exists on the same vector as the adjustable-threshold switch, or on a different vector. The input to the adjustable-threshold switch is an agent, or agents, (analogous to the exemplary protein X in the prokaryotic version) which modulates the ability of the fusion protein to activate transcription.

The generic scheme for constructing a eukaryotic adjustable-threshold switch construct is illustrated in Figure 18. Transcription of *Gene 1* from P_{min} is activated by a fusion protein composed of an O_A binding domain and an acidic activation domain. Activation by the activator protein may be positively or negatively modulated by a chemical signal (*Input*). P_{min} is simultaneously inhibited at operator site, O_2, by the bacterial repressor protein encoded by *Gene 2*. Promoter 2, P_2, efficiently transcribes *Gene 2* unless inhibited at operator site O_1 by the bacterial repressor protein encoded by *Gene 1*. Open arrows indicate direction of transcription. *Clone* is an additional gene or genes which may be placed under the control of P_{min} or P_2.

An exemplary construct containing the *tetO* operator sequence, the $P_{minhCMV}$ promoter, the P_{hCMV} promoter, the *lexA* gene, and the *lacI* gene is shown in Figure 19. Transcription of the *lexA* gene from the minimal Human Cytomegalovirus Immediate Early Promoter, P_{minCMV}, is activated by the HSV-VP16:TetR fusion protein which binds at the *tetO* operator site. The HSV-VP16:TetR fusion protein is synthesized from the P_{hCMV} promoter contained in a separate construct. Activation by the HSV-VP16:TetR can be positively or negatively modulated by a doxycycline, a tetracycline derived compound. P_{minCMV} is simultaneously

inhibited at operator site, O_{lac}, by the bacterial Lac repressor protein encoded by *lacI*. The constitutive promoter, P_{hCMV}, efficiently transcribes the *lacI* gene unless inhibited at operator site, O_{lex}, by the bacterial LexA repressor encoded by *lexA*. Open arrows indicate direction of transcription. *Clone* is an additional gene or genes which may be placed under the control of P_{minCMV} or P_{hCMV}. Furthermore, the HSV-VP16:TetR fusion protein may be designed such that doxycycline (a tetracycline derivative) acts as an inhibitor or, alternately, a co-activator of transcription initiation. Thus, this construct may be activated or inactivated by doxycycline.

Construction of eukaryotic multi-state oscillators as exemplified by a two-state oscillator is an extension of the same design principles discussed above. For example, one of ordinary skill in the art appreciates that a hybrid constitutive promoter or an inducible eukaryotic promoter must be inserted in the oscillator feedback loop.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

EXAMPLE 1

Design Considerations For Construction of pTAK1 Plasmid Carrying Exemplary Toggle Switches

This Example demonstrates the design considerations which were determined by the inventors to be implicated in designing an exemplary toggle switch.

All theoretical curves were calculated numerically from Equations 2 using Matlab (Mathworks), XPP-AUTO, software for simulation and analysis of differential equations (G.B. Ermentrout, University of Pittsburgh, available at http://www.pitt.edu/~phase/), or AUTO, a bifurcation package included in the XPP-AUTO software (E. Doedel, McGill University).

The bistability of the toggle arises from the mutually inhibitory arrangement of the repressor genes. In the absence of inducers, either one of the two promoters can transcribe its repressor gene, but they cannot simultaneously transcribe in a stable fashion. Thus, two stable states are possible: one in which promoter 1 transcribes repressor 2, and one in which promoter 2 transcribes repressor 1. Switching is accomplished by transiently

introducing an inducer of the currently active repressor. The inducer permits the opposing repressor to be maximally transcribed until it stably represses the originally active promoter.

The behaviour of the toggle switch and the conditions on bistability are predicted from Equations 2 described *supra*. The final form of the toggle Equations 2 preserves the two most fundamental aspects of the network: cooperative repression of constitutively transcribed promoters (the first term in each equation), and degradation/dilution of the repressors (the second term in each equation).

The parameters α_1 and α_2 are lumped parameters that describe the net effect of RNA Polymerase (RNAP) binding, open-complex formation, transcript elongation, transcript termination, repressor binding, ribosome binding and polypeptide elongation. The cooperativity described by β and γ can arise from the multimerization of the repressor proteins and the cooperative binding of repressor multimers to multiple operator sites in the promoter. An additional modification to Equations 2 is needed to describe induction of the repressors (see Figure 7 caption). Since bistability arises in the absence of inducers, this modification is not included in the present discussion.

The geometric structure of Equations 2, illustrated in Figures 2a and 2b, reveals the origin of the bistability: the nullclines ($du/dt = 0$ and $dv/dt = 0$ in Figure 2) intersect at three points, producing one unstable and two stable steady-states. From Figures 2a and 2b, three key features of the system become apparent. First, the nullclines intersect three times because of their sigmoidal shape, which arises for $\beta, \gamma > 1$. Thus, the bistability of the system depends on the cooperative repression of transcription. Second, the rates of synthesis of the two repressors must be balanced. If the rates are not balanced, the nullclines will intersect only once, producing a single stable steady-state. Third, the structure of the toggle network creates two basins of attraction. Thus, a toggle with an initial condition anywhere above the separatrix will ultimately settle to state 1, whereas a toggle starting below the separatrix will settle to state 2.

The conditions for a bistable toggle network are illustrated in Figures 3c and 3d. As the rates of repressor synthesis are increased, the size of the bistable region increases. Furthermore, the slopes of the bifurcation lines, for large α_1 and α_2, are determined by β and γ (Figure 3c). Figure 3d shows that reducing the cooperativity of repression (β and γ) reduces the size of the bistable region. Bifrucation lines are illustrated for three different values of β and γ. The bistable region lies inside of each pair of curves. Thus, to obtain bistability, at least one of the inhibitors must repress expression with cooperativity greater

than one. This suggests that repressor multimerization, or multiple operator sites in the promoter, is a necessary condition for bistability. Moreover, higher-order cooperativity will increase the robustness of the system, allowing weaker promoters to achieve bistability, thereby producing a broader bistable region.

For the construction of a toggle switch, the P_L promoter and *cIts* gene from the bacteriophage λ, the *lacI* gene from *E. coli*, the P_{trc} promoter (a fusion of the *trp* and *lac* promoters from *E. coli*) were chosen, and *E. coli* was selected as the host organism. These elements, arranged as shown in Figure 4, are chosen because they are well-characterized and readily obtainable, which facilitates any necessary modifications. The P_L and P_{trc} promoters are constitutive, i.e., they are efficiently transcribed by the RNAP alone, and are repressed by the *cIts* and *lacI* gene products, respectively. The *cI* gene, because it carries the temperature sensitive mutation, allows switching to P_L expression by heat shock. If the wild-type allele of the *cI* gene is used instead, then induction of P_L expression can be achieved through the use of nalidixic acid or UV light. On the other hand, switching the toggle to P_{trc} expression is accomplished by a pulse of IPTG.

Although previous studies have shown that the strengths of the two promoters (*i.e.*, P_L and P_{trc}) are reasonably well-matched [de Boer, HA, et al. (1983) Proc. Natl. Acad. Sci. USA 80:21-25; Rosenberg, M, et al. (1983) Methods in Enzymology 101:123-139] (*i.e.*, the strengths are within the bi-stable region illustrated in Figure 3A) modifications to one or both promoters will likely be necessary. Changes to either promoter are facilitated by the modular design of the toggle plasmid, pTAK. Figure 11 is a map of $pTAK_1$ with key restriction sites. To prevent errant transcription of either of the toggle genes, the two operons are placed back-to-back in opposite orientation. In addition, transcription terminators are placed immediately downstream of both operons. Although both genes and both promoters are included on the $pTAK_1$ plasmid, such a configuration is not required. The toggle remains functional if the P_L-*lacI* fragment and the P_{trc}-*cIts* fragment are on separate vectors transfected into the same host cell.

The sequences of the P_L and P_{trc} promoter regions are illustrated in Figure 12. Consensus sequences are darkly shaded and labeled. Operators and coding regions are lightly shaded and labeled. Restrictions enzyme recognition sequence are marked in boldface and labeled in bold italic font. Restriction sites surrounding and within the promoters allow easy excision and replacement of all or part of the promoter region. Thus, any of the key promoter elements, the -10 sequence, the -35 sequence, the UP sequence, the

RBS, or the mRNA leader sequence can be easily altered. Another key feature of the design is the use of identical sequences for the 17 bases upstream of the start codons of both genes. This region, which includes the RBS, ensures that the efficiency of translation initiation of both genes will be well-matched. Finally, the GTG start codon of the *lacI* gene was replaced with ATG to facilitate its subcloning.

Briefly, the growth dynamics of the *E. coli* host strains, CGSC 808 (*E. coli* Genetic Stock Center) and JM105 (Pharmacia) are characterized. Each host is also characterized for its tolerance to extended overexpression of a foreign protein.

Next, four plasmids which are used to make pTAK1 are constructed using standard recombinant DNA methods: (1) pOS_1, containing the P_{trc}-*cIts* construct, (2) pOS_1-GFPuv, containing the P_{trc}-*cIts*-GFPuv construct, (3) pOS_2, containing the P_L-*lacI* construct, and (4) pOS_2-GFPuv, containing the P_L-*lacI*-GFPuv construct as outlined in Figures 13 and 14. Briefly, three plasmids are used in this process: pTrc99a (Pharmacia), pGW7 (ATCC), and pGFPuv (Clontech) which contains the sequence encoding green fluorescent protein (GFP). GFPuv is selected as the gene of interest to be cloned into the multiple cloning site (MCS) of $pTAK_1$ in order to facilitate detection of switching by observing fluorescence by GFP. Plasmids pOS_1-GFPuv and pOS_2-GFPuv offer the advantage of fluorescence-based quantitative assays of promoter strength.

The strength of the P_{trc} and P_L promoters is tested by expressing pOS_1-GFPuv and pOS_2-GFPuv in the *E. coli* cells. The strength of inhibition of the promoters by the Lac repressor (product of the *lacI* gene) and the λ Repressor (product of the *cIts* gene) is tested using methods known in the art. The promoter sequence are modified, if necessary, and retested until the promoter strengths are within the bounds for bi-stability predicted by the model in Equations 1.

The $pTAK_1$ plasmid which contains the complete toggle switch construction is constructed as illustrated in Figures 13-15. A multiple cloning site exists downstream of the EcoRI site between the *lacI* gene and the *rrn*T_1T_2 terminator on $pTAK_1$. This MCS's can be used to place any gene under the control of the toggle switch. The $pTAK_1$ plasmid is tested for bi-stable behavior in CGSC 808 and the promoter strengths are modified as necessary to obtain a balance between effective bi-stable behavior and lethality due to overexpression.

EXAMPLE 2

Implementation Of Design Considerations For Construction Of Five Exemplary Toggle Switches

This Example demonstrates the successful construction, in conformance with the invention's design considerations described *supra*, of five toggle switches which exhibit the bi-stability and "perfect" switching thresholds predicted by the inventors for the toggle switch.

All toggle switches were implemented on *E. coli* plasmids conferring ampicillin resistance and containing the pBR322 ColE1 replication origin. As illustrated in Figure 20, the toggle switch is composed of two repressors and two constitutive promoters. Each promoter is inhibited by the repressor transcribed by the opposing promoter. Although Figure 1 illustrates the toggle with a reporter gene placed downstream of the second promoter, a second reporter could easily be placed downstream of the first promoter. Bistability is possible with any set of promoters and repressors as long as they fulfill a minimum set of conditions, as described *supra*.

The toggle switch genes were arranged as a Type IV plasmid as shown in Figure 21. Promoters are marked by solid rectangles with arrowheads. Genes are denoted with solid rectangles. Ribosome binding sites and terminators (T_1T_2) are denoted by outlined boxes. The Ptrc-2 promoter with RBS-E and the *lacI* gene are used in all Type II, III and IV plasmids. RBS-B is used for the reporter gene in all Type IV plasmids. Different P1 promoters, RBS1 ribosome binding sites, and/or R1 repressors, are used for the various toggle switches. The two opposing promoters and repressor genes were arranged back-to-back in opposite orientation to minimize unintended phenomena such as transcription read-through and antisense transcription. Though all genes were contained on a single plasmid, the two halves of the toggle could, in principle, be placed on separate plasmids without altering the functionality of the toggle.

i. Strains, Growth Conditions, Chemicals

The host cell for all promoter assays and toggle switch experiments was *E. coli* strain JM2.300 [λ-, *lacI22*, *rpsL135* (StrR), *thi*-1] (CGSC strain 5002). JM2.300, which contains few mutations, is a fast growing strain that can tolerate enormous overexpression of plasmid-bound genes. Because JM2.300 contains no λ repressor and carries a

non-functional Lac repressor (*lacI22*), it is considered by the inventors to be an ideal host for the toggle switch.

All cells were grown in LB medium (Difco) with 100 μg/ml ampicillin plus inducers as indicated in the text. All Type I and pIKE series plasmids were grown at 37±1°C unless otherwise indicated. All pTAK series plasmids were grown at 32±1°C except during thermal induction. Thermal induction was carried out at 42±1°C, unless otherwise indicated. For all expression tests, cells were maintained in logarithmic growth phase by periodic 500-1000 fold dilution into fresh medium.

Ampicillin and IPTG were purchased from Sigma. Anhydrotetracycline was purchased from ACROS Organics. All other chemicals were obtained from Fisher.

ii. Plasmid Construction

Two classes of toggle switches were constructed. Both classes use the Lac repressor (*lacI*) in conjunction with the Ptrc-2 promoter for the first promoter-repressor pair. For the second promoter-repressor pair, class 1 plasmids (pTAK series) use the P_Ls1con promoter in conjunction with a temperature-sensitive mutant of the λ repressor (*cIts*). Class 1 plasmids were switched between states by a pulse of IPTG or thermal induction. Class 2 plasmids (pIKE series) use the $P_L$tetO-1 promoter in conjuction with the TetR repressor as the second promoter-repressor pair. Class 2 plasmids were switched between states by a pulse of IPTG or a pulse of anhydrotetracycline (aTc). In total, four variants of the class 1 toggles and two variants of the class 2 toggles were constructed.

Plasmids were constructed using basic molecular cloning techniques described in standard cloning manuals [Ausubel *et al.* in *Current Protocols in Molecular Biology* (Wiley, New York, 1987); Sambrook *et al.* in *Molecular Cloning: A Laboratory Manual.* (Cold Spring Harbor Laboratory Press, Plainview, NY, 1989)]. Restriction enzymes were purchased from New England Biolabs and Promega; Pfu*Turbo* polymerase was purchased from Stratagene; all other enzymes were purchased from New England Biolabs; all synthetic oligonucleotides were purchased from Operon Technologies. All genes, promoters and transcription terminators were obtained by PCR amplification using Pfu*Turbo* proofreading polymerase and synthetic primers with overhanging ends containing the appropriate restriction sites. Ribosome binding sites were included in the overhanging ends of the primers. Site mutations were performed using either the Stratagene QuickChange or ExSite protocols.

Genes, promoters and transcription terminators were obtained as follows: Ptrc-2 from pTrc99a (AP Biotech), P_L from pXC46 (ATCC), P_1tetO-1 by total synthesis according to the published sequence [Lutz & Bujard (1997) Nucleic Acids Res. 25:1203-1210], lacI from pTrc99a, *cIts* from pGW7 (ATCC), *tet*R from pcDNA6/TR (Invitrogen), *gfpuv* from pGFPuv (Clontech), *gfpmut3* from pJBA111 (gift of J.B. Andersen, Technical University of Denmark), and rrnT1T2 terminators from pTrc99a. All plasmids contained the ampicillin resistance region and ColE1 origin of replication from the pTrc99a plasmid. All cloning was performed by TSS transformation [Ausubel *et al.* in *Current Protocols in Molecular Biology* (Wiley, New York, 1987)] into either *E. coli* strain JM2.300 (CGSC), JC158 (CGSC), or TAP106 (ATCC).

DNA sequencing was performed using a Perkin-Elmer ABI Prism 377 Sequencer.

In all toggle plasmids, the *gfpmut3* reporter gene was arranged as the second cistron downstream of the Ptrc-2 promoter. Thus, transcription from Ptrc-2 (and repression of P1) results in the expression of GFPmut3. For clarity, this state is termed the "high" state. The opposing state, in which P1 is transcribed and Ptrc-2 is repressed, is termed the "low" state. Unless otherwise indicated, GFPmut3 is the reporter used in all plasmids. Gfpmut3, a mutant of wild-type GFP containing S65G and S72A substitutions, is optimized for flow cytometry [Cormack *et al.* (1996) Gene 173:33-38]. The inventors' tests showed it to be 50-70 times brighter than GFPuv when expressed in *E. coli* and assayed in a FACSCalibur flow cytometer.

The structures of the three promoters used in the toggle are illustrated in Figure 22. In Figure 22a, the upstream limit of each promoter is marked by the indicated restriction site. Operator sites are marked be a single underbracket. The initiation of transcription is denoted with arrows. SD denotes the Shine-Dalgarno sequence. Mutations in the -10 sequence of P_Ls1con are indicated with lowercase letters. In Figure 22 b, the Shine-Dalgarno sequences and start codons are in boldface. Sequences are ranked in order of their translational efficiency (A = highest, G = lowest). Bases -48 to +27 of the Ptrc promoter, where +1 is the initiation of transcription, were amplified by PCR from pTrc99a to form the Ptrc-2 promoter. Ptrc-2 is a highly efficient fusion of the Ptrp and Plac promoters and is nearly identical to the commonly used Ptac promoter. P_Ls1con is a shortened version of the wild-type P_L promoter with additional mutations conferring a consensus -10 sequence. P_Ls1con was amplified from bases -75 to the Shine-Dalgarno sequence of pXC46. Thus P_Ls1con eliminates the P_{L2} secondary promoter and the L1 and

L2 integration host factor binding sites of the wild-type P_L promoter [Giladi *et al.*(1992) J. Mol. Biol. 224:937-948]. Elimination of P_{L2}, L1, L2 and introduction of the -10 mutations serve to weaken the native strength of the extremely strong P_L promoter while retaining all three operators for λ repressor binding. The $P_L$tetO-1 promoter, obtained through total synthesis according to the published sequence [Lutz & Bujard (1997) Nucleic Acids Res. 25:1203-1210], contains two copies of the O2 operator of the Tn10 tetracycline resistance operon---one between the consensus -35 sequence and the -10 sequence of P_L, and one upstream of the -35 sequence. The $P_L$tetO-1 promoter was substantially less efficient than both Ptrc-2 and P_Ls1con, but it was effectively repressed by the wild-type TetR repressor. The ranked order of the transcriptional efficiencies of the promoters is: P_Ls1con > Ptrc-2 > $P_L$tetO-1.

In all plasmids, the sequence of the three promoters was unchanged. Any modifications in the rates of synthesis of the repressors (α_1 and α_2 in the model) or the reporter genes were achieved by exchanging the downstream ribosome binding sites (RBS). The RBS sequences used in this work are illustrated in Figure 22 in order of efficiency.

iii. Assay of Gene Expression And Promoter Strength

All expression data were collected using a Becton-Dickinson FACSCalibur flow cytometer with a 488nm argon excitation laser and a 515-545nm emission filter. Prior to assay, cells were pelleted and resuspended in 0.22μm filtered PBS (58mM Na_2HPO_4, 17mM NaH_2PO_4, 68 mM NaCl, pH=7.4). Cells were assayed at low flow rate and fluorescence was calibrated using InSpeck Green fluorescent beads (Molecular Probes). All measurements of gene expression were obtained from three independent cultures maintained simultaneously under identical conditions. For each culture, 40,000 events were collected. All flow data were converted to ASCII format using MFI (E. Martz, University of Massachusetts, Amherst, available at http://marlin.bio.umass.edu/ mcbfacs/flowcat.html\#mfi) and analyzed with Matlab (Mathworks).

The absolute strengths, in calibrated fluorescence units, of the promoter/RBS pairs used to construct the toggle switches are listed in Table 5. Measurements of promoter strengths were performed using Type I plasmids (Figure 21) and assays were performed as described above. Leakage expression from the promoters under fully repressed conditions is also listed in Table 5.

Table 5
Gene Expression by Plasmids

PLASMID	TYPE	P1	RBS1	RBS2	GFP EXPRESSION
Bare Promoters					
pMKN7a*	I	Ptrc-2	E	-	732 ± 108
pBAG102	I	$P_L$tetO-1	C	-	5.5 ± 0.1
pBAG103	I	$P_L$tetO-1	A	-	660 ± 42
pBRT21.1*	I	P_Ls1con	D	-	9,390 ± 840
pBRT21.1*†	I	P_Ls1con	D	-	14,300 ± 400
pBRT123	I	P_Ls1con	G	-	387 ± 21
pBRT124	I	P_Ls1con	F	-	972 ± 43
pBRT125	I	P_Ls1con	H	-	15.9 ± 3.2
lacI Repression					
pTAK102	II	P_Ls1con	D	-	36.0 ± 3.8
pTAK103a	II	P_Ls1con	G	-	137 ± 8
cI Repression					
pTAK106	III	P_Ls1con	D	-	2.5 ± 0.3
pTAK107	III	P_Ls1con	G	-	2.0 ± 0.1
tetR Repression					
pIKE108	III	$P_L$tetO-1	A	-	5.8 ± 1.0
pIKE110	III	$P_L$tetO-1	C	-	2.3 ± 0.2
Toggles					
pTAK117	IV	P_Ls1con	D	B	bistable
pTAK130	IV	P_Ls1con	G	B	bistable
pTAK131	IV	P_Ls1con	F	B	bistable
pTAK132	IV	P_Ls1con	H	B	bistable
pIKE105	IV	$P_L$tetO-1	A	B	monostable
pIKE107	IV	$P_L$tetO-1	C	B	bistable

* Estimated from flow-cytometer assay of GFPuv-expressing promoters.
† Grown at 32°C.

The efficacy of repression was tested using Type II plasmids (for Lac repression) or Type III plasmids (for λ or TetR repression). The efficacy of the three repressors, as used in the

toggle switches, can be inferred by comparing the strength of the bare promoters in Type I plasmids to their leakage expression under repressed conditions. The extremely efficient λ repressor, expressed from Ptrc-2-E, achieves ~6000 fold repression of the P_Ls1con-D promoter. On the other hand, the TetR repressor, also expressed from Ptrc-2-E, achieves only ~100 fold repression of the $P_L$tetO-1-A promoter.

iv. Demonstration of Bi-Stability

In order to examine the limits on the bistability of the toggle switch, the α_1 parameter space was experimentally scanned by inserting RBS1 sequences of varying efficiency into the class 1 and class 2 toggles. Four pTAK series plasmids (class 1) were constructed with RBS1 sequences D,F,G and H, and two pIKE series plasmids (class 2) were constructed with RBS1 sequences A and C. All four pTAK plasmids exhibited bistability, while only one pIKE plasmid (pIKE107) exhibited bistability.

The demonstration of bistability is illustrated in Figure 23. In this experiment, the toggle and control plasmids were grown in *E. coli* strain JM2.300 for 23.5 hours. At 6, 11, 18 and 23.5 hours, samples were taken and cells were pelleted, washed once in LB or PBS, and diluted 500-1000 fold into fresh medium with or without inducers as appropriate. Cells were initially grown for 6 hours with 2mM IPTG, inducing GFPmut3 expression in all toggles and the IPTG-inducible pTAK102 control plasmid. The thermally-inducible pTAK106 control and the aTc-inducible pIKE108 control did not express GFPmut3. Cells were washed and diluted into fresh medium with no IPTG and grown an additional 5 hours. The five bistable toggle plasmids, which had been switched to the high state, continued to express GFPmut3 in the absence of inducer, while the pTAK102 control plasmid and the monostable pIKE105 toggle plasmid, returned to the low state. Cells were diluted into fresh medium and induced at 42°C (pTAK plasmids only) or grown in the presence of 500 ng/ml aTc (pIKE plasmids only). After 7 hours growth, GFPmut3 expression in all toggles had been shut off, while GFPmut3 expression in the thermally-inducible pTAK106 control and the aTc-inducible pIKE108 control was up-regulated. Cells were washed and diluted into fresh medium with no inducers and returned to standard temperature. After an additional 5.5 hours, the five bistable toggle plasmids, which had been switched to the low state, continued to repress GFPmut3 expression, while the pTAK106 and pIKE108 controls returned, as expected by the inventors, to their non-induced condition.

In Figure 23c, the long-term stability of the two states of the pTAK117 toggle plasmid is illustrated. In this experiment, a single culture of pTAK117 cells (initially in the low state) was divided into two groups and diluted. The first group was grown in medium with no inducers while the second group was grown in medium plus 2mM IPTG. After 6 hours, cells were pelleted, washed once in LB and diluted 1000 fold into fresh medium with no inducer. Both groups of cells were grown for an additional 22 hours while taking samples and diluting into fresh medium every 6 - 8.5 hours. For the entire 22 hours of growth, the two groups of pTAK117 cells remained in their initial high or low states.

Although all of the toggle plasmids contain the same configuration of elements, one plasmid, pIKE105, did not exhibit bistability. Without limiting the invention to any particular mechanism, the inventors suggest the following qualitative explanation (illustrated in Figure 24) for the behaviour of the toggle plasmids. In Figure 24, the class 1 bifurcation region and toggles are denoted by continuous lines and crosses, respectively. The class 2 bifurcation region and toggles are denoted by hatched lines and circles, respectively. The positions of the bifurcation curves and plasmids are qualitative estimates. The pTAK series plasmids contain the λ repressor, which dimerizes and binds cooperatively to three operator sites, and the Lac repressor, which forms a tetramer before binding to its operator site. Thus, both repressors should exhibit high cooperativity in the repression of their corresponding promoters (β and λ large) and hence, produce a broad bistable region. The pTAK117 plasmid, with the extremely strong P_Ls1con-D promoter, likely exists somewhere near the edge of the bistable region. All other pTAK toggles contain weaker P_Ls1con promoters. Thus, the effective rate of Lac repressor synthesis (α_1) was reduced and the toggles were shifted closer to the center of the bistable region. Likewise, the pIKE105 toggle, which contains the weaker $P_L$tetO-1-A promoter, synthesizes Lac repressor at a lower rate than pTAK117. At the same time, α_2 was increased moderately because the TetR repressor was shorter, and thus more efficiently transcribed, and more tightly binding to its operator sites ($K_d \approx 10^{-11}$M for TetR to Otet2 [Hillen & Berens (1994) Annu. Rev. Microbiol. 48:345-369] versus $K_d \approx 10^{-9}$M for λ to O_{L1}-O_{L3} [Johnson *et al.* (1981) Nature 294:217-223]. Thus, one would expect the pIKE105 toggle to move further into the bistable region. However, the TetR dimer binds non-cooperatively to only two operator sites, while the λ dimer binds cooperatively to three operators. Thus, the exponent γ was reduced, the bistable region narrows, and the pIKE105 plasmid falls in the region of low state monostability. When the strength of the $P_L$tetO-1 promoter was reduced by replacing

RBS-A with RBS-C, as in pTAK107, the value of α_1 was reduced and the plasmid was shifted back into the bistable region.

v. Generation of "Perfect" Switching Thresholds

Another qualitative prediction of the toggle model is that a genetic toggle will have nearly ideal switching thresholds due to the existence of saddle-node bifurcations. (Bifurcations occur at the transition between monostability and bistability.) Switching of the toggles by IPTG, aTC or heat induction alters the geometric structure of the toggle such that it is pushed through a bifurcation (the switching threshold) into monostability of the opposing state. Once the toggle crosses that threshold, it immediately switches to maximal expression in the opposing state. This ideal threshold, or bifurcation, demonstrated experimentally in the pTAK117 toggle switch, is illustrated in Figure 25.

Figure 25a shows the steady-state gene expression after 17 hour induction. The pTAK117 toggle plasmid (circles) exhibits a quasi-discontinuous jump to the high state while the pTAK102 control plasmid (triangles) exhibits a sigmoidal induction curve. Point 1 is taken from separate experiments measuring the high state of pTAK117 with no inducer. Points 3a and 3b are the high and low modes of a bimodally distributed cell poplulation. The bimodality occurs due to natural fluctuations in gene expression and the close proximity of the toggle switch to its bifurcation point. Theoretical curves are calculated from Equations 1 with the term $\mu/(1 + [\mathrm{IPTG}]/K)^{\eta}$, where K is the dissociation constant of IPTG from LacR and η is the cooperativity of IPTG binding, replacing μ in the denominator of Equations 1b. The solid line curve shows the stable steady-states and the discontinuous curve shows the unstable steady-state of the toggle. The hatched curve shows the steady-state of the IPTG-inducible control plasmid. Model parameters for the theoretical curves are: $\alpha_1 = 156.25$, $\alpha_2 = 15.6$, $\beta = 2.5$, $\gamma = 1$, $\eta = 2.0015$, $K = 2.9619 \times 10^{-5}$. Figure 25 b shows the fraction of toggle cells in the high state at various concentrations of IPTG. The dramatic switching to the high state is more clearly visible. High and low cell populations were divided as described for panel c. Figure 25c shows scatter plots (left plots) and histograms (right plots) illustrating the condition of the toggle cells at points 2, 3 and 4 (of panel a) near the bifurcation point. High-state and low-state cell populations are divided by the red line in the scatter plots. The two states of the toggle are clearly evident in the bimodally distributed cells (point 3).

In this experiment, pTAK117 (initially in the low state) and pTAK102 (as a control) were grown in 13 different concentrations of IPTG for 17 hours, diluting twice (at 6 and 12.5 hours) into fresh medium with the same IPTG concentration. The cells were grown for this length of time in order to ensure that they reached steady-state expression levels.

Induction of the pTAK102 control has the familiar sigmoidal shape. In contrast, the pTAK117 toggle follows the induction curve of pTAK102 up to an IPTG concentration of 40μM, at which point it exhibits a quasi-discontinuous jump to the high expression state. This discontinuity is the saddle-node bifurcation.

Due to the natural fluctuations in gene expression, the bifurcation is not a perfect discontinuity as predicted by the deterministic toggle equations. The stochastic nature of gene expression causes variability in the location of the switching threshold and thus blurs the bifurcation point. Near the bifurcation, this blurriness was realized as a bimodal distribution of cells (Figure 25c).

vi. Switching Time

The switching time of the pTAK117 plasmid from the low to high states and from the high to low states is illustrated in Figure 26. In this experiment, pTAK117 cells initially in the low state were diluted in fresh medium and induced with 2mM IPTG. Separate cultures were grown for 35 minutes to 6 hours before pelleting, washing, and diluting the cells 500 fold in fresh medium with no inducer. Growth was continued until 10.25 hours after the start of the experiment and cells were assayed in the flow cytometer. Conversely, pTAK117 cells initially in the high state were diluted in fresh medium with no inducer. Separate cultures were grown at 41±1°C for 35 minutes to 6 hours before diluting the cells 500 fold in fresh medium with no inducer. Growth was continued at standard temperature until 10.25 hours after the start of the experiment and cells were assayed in the flow cytometer.

In Figure 26 panels a and b, the fraction of cells in the high state is plotted as a function of the induction time. Cells were divided between high and low states as explained for panel c of Figure 25. Figure 26c shows switching of pTAK117 cells from the low to high state by IPTG induction. The cell population at four time points is illustrated. Cells begin switching between 3 and 4 hours as evidenced by the appearance of a bimodal distribution. The switching is complete by 6 hours.

As evidenced by the appearance of a bimodal distribution at 4 hours (Figure 26), the pTAK117 plasmid began switching to the high state between 3 and 4 hours of IPTG induction. By five hours the switching was nearly complete and by 6 hours it was complete. On the other hand, switching from the high state to the low state was completed in 35 minutes or less.

The primary determinant of switching time is the rate of destruction of the expressed transcribed repressor protein. The concentration of initial repressor must be reduced sufficiently to allow the system to cross into the basin of attraction of the opposing stable state. The principal factor governing Lac repressor destruction is dilution due to cell growth. Since doubling time of the JM2.300 cells, when expressing in the high state, was 38 minutes, the switching time from low to high was on the order of hours. On the other hand, switching from high to low was accomplished by thermal destabilization of the temperature-sensitive λ repressor. Thermal denaturation of the λ repressor was on the order of a few minutes. Thus, switching to the low state was substantially more rapid than switching to the high state. Furthermore, the configuration of the pTAK117 plasmid (the rate of Lac repressor synthesis was more than an order of magnitude higher than the rate of λ repressor synthesis) suggests that the low state was more stable (i.e., it has a larger basin of attraction) than the high state.

EXAMPLE 3

Design and Construction of a Plasmid Carrying An Exemplary Adjustable-Threshold Switch

The experimental methods used to construct and test adjustable-threshold switch constructs parallel those discussed above for the toggle switch construct (Example 1). Construction of the exemplary adjustable-threshold switch requires a promoter that is activated by a regulatory protein. Additionally, expression from this promoter is preferably negligible in the absence of the activator. Finally, this promoter must be simultaneously suppressed by the opposing gene in the switch construct (*i.e.*, *Gene 2* in Figure 5). Construction of a promoter that satisfies all of the above requirements is facilitated by the modular structure of the P_{trc} promoter used in Example 1. The Lac repressor binding site begins exactly at the first nucleotide of the mRNA transcript. The complete P_{trc} promoter, including all of the RNAP recognition sites, is located upstream of the Lac repressor binding

site. Thus, the entire P_{trc} promoter upstream of the +1 nucleotide may be removed and replaced by nearly any positively regulated promoter element, such as the promoters of Table 2. The new hybrid promoter, which retains the Lac repressor binding site, is thus both positively and negatively regulated. For example, the P_{bad} promoter, which is activated by the ARAC protein in the presence of arabinose, is fused to the O_{lac} operator region of P_{trc}. The resulting hybrid promoter is positively activated by ARAC-arabinose and repressed by lacI (Figure 27).

The opposing promoter may remain unaltered, or its strength may be modified in order to adjust the threshold location or hysteresis. Such strength modifications may also be necessary for the hybrid activator/repressor promoter. The modifications can be introduced through standard recombinant DNA techniques.

Dynamic adjustment of the threshold concentration of the inducing agent exemplified by protein X in Figure 5 is also possible. Because the strengths of the promoters (α_1 and α_2) are dependent on the strength of repressor-DNA binding, an inducer compound such as IPTG can be used to alter the promoter strength. The inducer, by competitively binding the repressor, effectively raises the dissociation constant of the repressor-DNA binding. Thus, by adjusting the concentration of inducer in the medium, the threshold can be dynamically altered.

Many suitable activatable promoters are known in the art. These and their cognate activators are exemplified by the activators/promoters listed in Table 2.

EXAMPLE 4

Design and Construction of Two Plasmids Each Carrying An Exemplary Two-State Oscillator

The methods used to construct and test two-state oscillators parallel those outlined for the toggle switch construct of Example 1. The design of a two-state oscillator is an extension of the adjustable-threshold switch of Example 3. The output of the switch (i.e., the expression of one of the two genes) is fed back into the input of the switch (i.e., the activator protein concentration) with a time delay. There are two methods to achieve this feedback.

The first method, as illustrated in Figure 9A, is to place the expression of the activator (protein X) under the indirect control of promoter 2. The single level of

indirection, provided by *Gene 3* and promoter 3, is necessary to produce the required delay. Thus, promoter 2 directs the transcription of two genes: one represses promoter 1, while the other activates promoter 3.

In the second method, illustrated in Figure 9B, promoter 3 is replaced with a constitutively transcribed promoter that is repressed by any product of promoter 1.

For both the first and second methods, activators and regressors which are suitable for the feedback genes are known in the art (Tables 1 and 2). Modification of these elements is likely to be necessary to achieve the desired time delay. For example, the time delay can be increased either by decreasing the rate of degradation of the protein X or by increasing the rate of degradation of the proteins encoded by Genes 1, 2 and 3 in the first method and Genes 1 and 2 in the second and third methods.

From the above, it is clear that the invention provides compositions for regulating gene expression and in particular for investigating the interaction of multiple genes.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art and in fields related thereto are intended to be within the scope of the invention.

CLAIMS

1. A method of altering transcription of a gene of interest, comprising:
   (a) providing:
      (i) a host cell;
      (ii) a composition comprising a first construct and a second construct, wherein:
         (a) said first construct comprises a first constitutive promoter operably linked to a first gene encoding a first protein and to a first gene of interest; and
         (b) said second construct comprises a second constitutive promoter operably linked to a second gene encoding a second protein, wherein said first protein represses transcription from said second promoter and said second protein represses transcription from said first promoter;
      (iii) a first agent capable of reducing repression of transcription from said first promoter; and
      (iv) a second agent capable of reducing repression of transcription from said second promoter;
   (b) transfecting said host cell with said composition;
   (c) exposing said transfected cell in any order to:
      (i) said first agent such that transcription of said first gene of interest is induced compared to transcription in the absence of said first agent; and
      (ii) said second agent such that transcription of said first gene of interest is repressed compared to transcription in the absence of said second agent.

2. The method of Claim 1, wherein said exposing is transient.

3. The method of Claim 1, wherein said host cell is prokaryotic.

4. The method of Claim 3, wherein said prokaryotic cell is *Escherichia coli*.

5. The method of Claim 1, wherein said host cell is eukaryotic.

6. The method of Claim 5, wherein said eukaryotic host cell is selected from the group consisting of yeast and human.

7. The method of claim 1, wherein said second construct further comprises a second gene of interest operably linked to said second constitutive promoter, said exposing of said transfected cell to said first agent results in repressed transcription of said second gene of interest compared to transcription in the absence of said first agent, and said exposing of said transfected cell to said second agent results in induced transcription of said second gene of interest compared to transcription in the absence of said second agent.

8. A method of increasing transcription of a gene of interest, comprising:

(a) providing:

(i) a host cell;

(ii) a composition comprising a first construct and a second construct, wherein:

(a) said first construct comprises an inducible promoter operably linked to a first gene encoding a first protein and to a gene of interest; and

(b) said second construct comprises a constitutive promoter operably linked to a second gene encoding a second protein, wherein said second protein represses transcription from said first promoter and said first protein represses transcription from said second promoter;

(iii) an agent capable of activating transcription from said first promoter in said composition;

(b) transfecting said host cell with said composition; and

(c) exposing said host cell to at least a threshold concentration of said agent such that transcription of said gene of interest is increased compared to transcription in the presence of said agent at a concentration below said threshold concentration.

9. The method of Claim 8, wherein said host cell is prokaryotic.

10. The method of Claim 9, wherein said prokaryotic host cell is *Escherichia coli.*

11. The method of Claim 8, wherein said host cell is eukaryotic.

12. The method of Claim 11, wherein said eukaryotic host cell is selected from the group consisting of yeast and human.

13. A method of increasing transcription of a gene of interest, comprising:
   (a) providing:
      (i) a host cell;
      (ii) a composition comprising a first construct and a second construct, wherein:
         (a) said first construct comprises an inducible promoter operably linked to a first gene encoding a first protein; and
         (b) said second construct comprises a constitutive promoter operably linked to a gene of interest and to a second gene encoding a second protein, wherein said second protein represses transcription from said first promoter and said first protein represses transcription from said second promoter;
      (iii) an agent capable of activating transcription from said first promoter in said composition;
   (b) transfecting said host cell with said composition; and
   (c) exposing said host cell to a concentration equal to or lower than a threshold concentration of said agent such that transcription of said gene of interest is increased compared to transcription in the presence of said agent at a concentration greater than said threshold concentration.

14. The method of Claim 13, wherein said host cell is prokaryotic.

15. The method of Claim 14, wherein said prokaryotic host cell is *Escherichia coli.*

16. The method of Claim 13, wherein said host cell is eukaryotic.

17. The method of Claim 16, wherein said eukaryotic host cell is selected from the group consisting of yeast and human.

18. A method of expressing a gene of interest, comprising:

(a) providing:

(i) a host cell;

(ii) a composition comprising a first construct, second construct and third construct, wherein:

(a) said first construct comprises a first inducible promoter operably linked to a first gene encoding a first protein and to a gene of interest; and

(b) said second construct comprises a constitutive promoter operably linked to a second gene encoding a second protein and to a third gene encoding a third protein;

(c) said third construct comprises a second inducible promoter operably linked to a fourth gene encoding a fourth protein, wherein:

(i) said first protein represses transcription from said constitutive promoter;

(ii) said second protein represses transcription from said first inducible promoter;

(iii) said third protein increases transcription from said third promoter; and

(iv) said fourth protein increases transcription from said first inducible promoter;

(b) transfecting said host cell with said composition; and

(c) culturing said transfected cell such that said gene of interest is expressed.

19. The method of Claim 18, wherein said expression is periodic.

20. The method of Claim 18, wherein said host cell is prokaryotic.

21. The method of Claim 13, wherein said host cell is eukaryotic.

22. A method of expressing a gene of interest, comprising:

(a) providing:

(i) a host cell;

(ii) a composition comprising a first construct, second construct and third construct, wherein:

(a) said first construct comprises an inducible promoter operably linked to a first gene encoding a first protein, a second gene encoding a second protein, and to a gene of interest; and

(b) said second construct comprises a constitutive promoter operably linked to a third gene encoding a third protein; and

(c) said third construct comprises a third promoter operably linked to a fourth gene encoding a fourth protein, wherein:

(i) said first protein represses transcription from said constitutive promoter;

(ii) said second protein represses transcription from said third promoter;

(iii) said third protein represses transcription from said inducible promoter; and

(iv) said fourth protein increases transcription from said inducible promoter;

(b) transfecting said host cell with said composition; and

(c) culturing said transfected cell such that said gene of interest is expressed.

23. The method of Claim 22, wherein said expression is periodic.

24. The method of Claim 22, wherein said host cell is prokaryotic.

25. The method of Claim 22, wherein said host cell is eukaryotic.

26. A method of expressing a gene of interest, comprising:
   (a) providing:
      (i) a host cell;
      (ii) a composition comprising a first construct, second construct and third construct, wherein:
         (a) said first construct comprises an inducible promoter operably linked to a first gene encoding a first protein and to a gene of interest;
         (b) said second construct comprises a first constitutive promoter operably linked to a second gene encoding a second protein; and
         (c) said third construct comprises a second constitutive promoter operably linked to a third gene encoding a third protein, wherein:
            (i) said first protein represses transcription from said first and second constitutive promoters;
            (ii) said second protein represses transcription from said inducible promoter; and
            (iii) said third protein increases transcription from said inducible promoter;
   (b) transfecting said host cell with said composition; and
   (c) culturing said transfected cell such that said gene of interest is expressed.

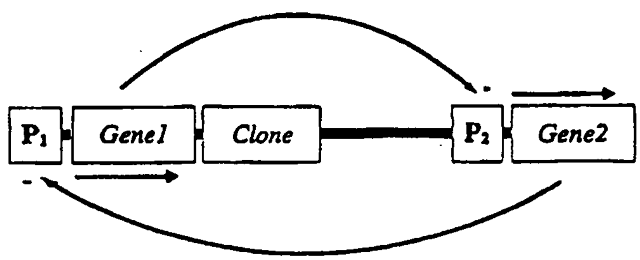
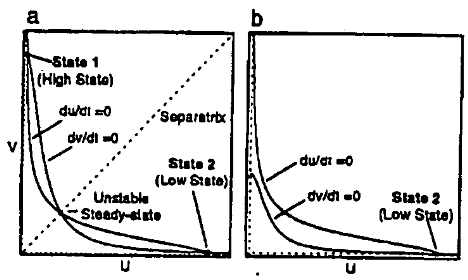


Figure 1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dv/dt =0
Separatrix
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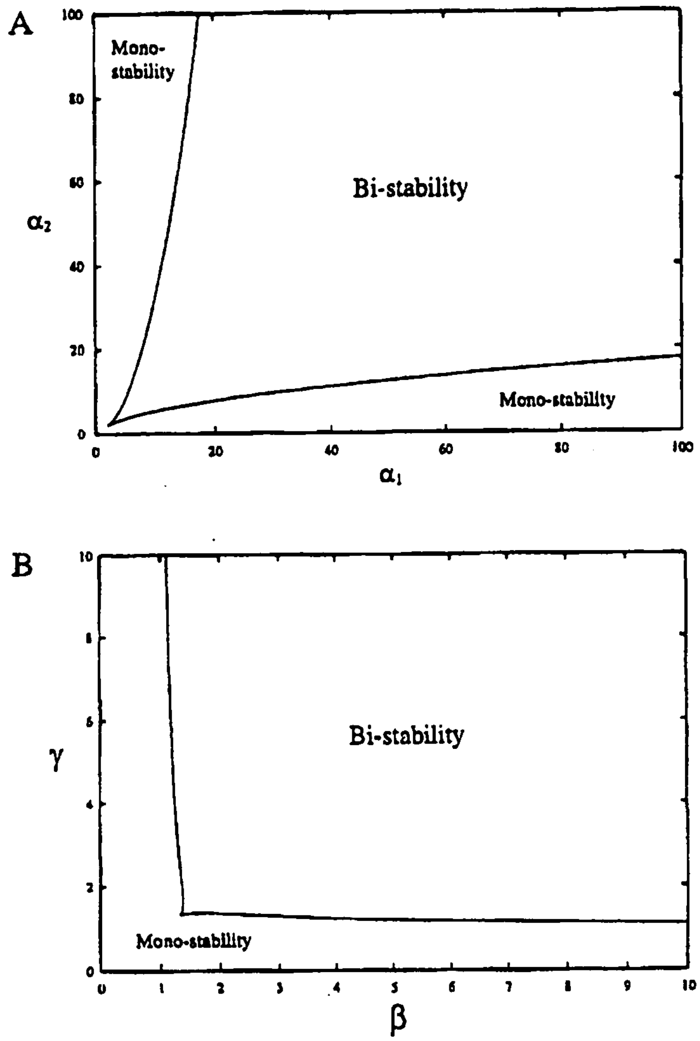
dv/dt =0
State 2
(Low State)
u

Figure 2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Bi-stability
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60
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γ
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5
6
7
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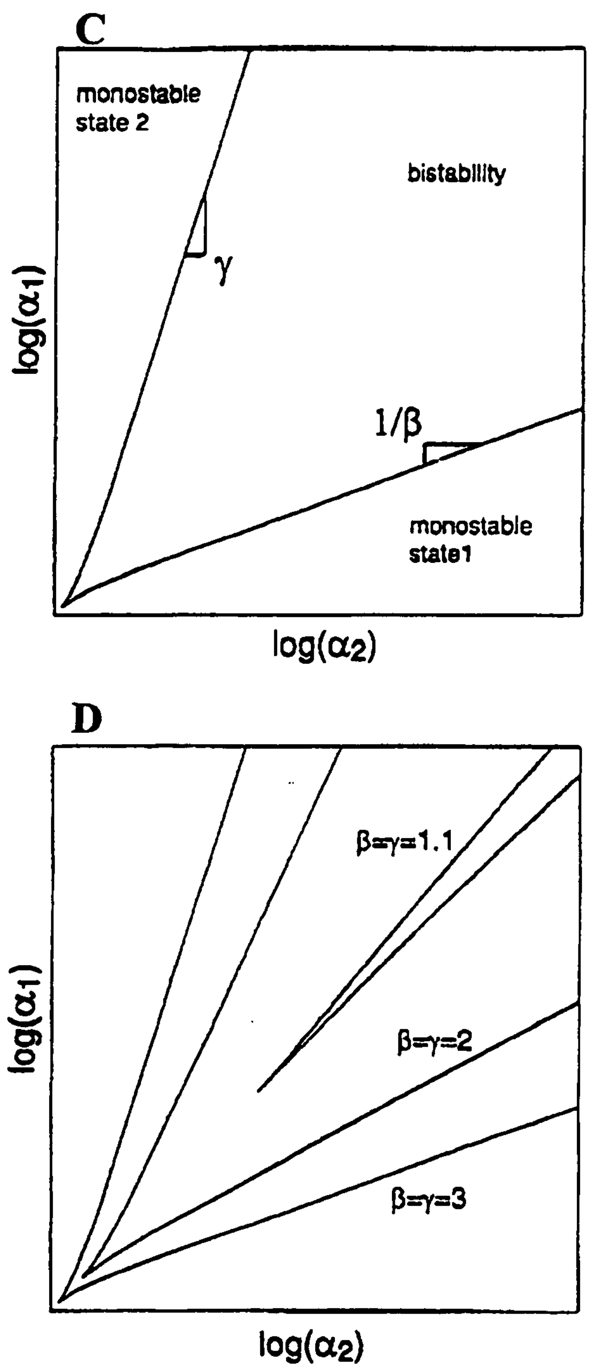
10

Figure 3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γ
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log(α1)
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β=γ=2
β=γ=3
log(α1)
log(α2)

Figure 3

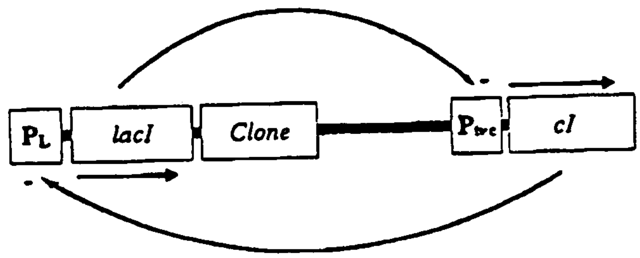


Figure 4

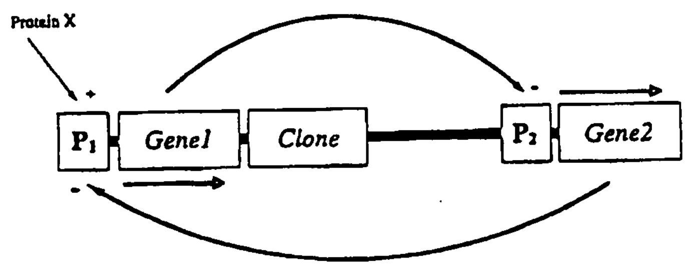


Figure 5

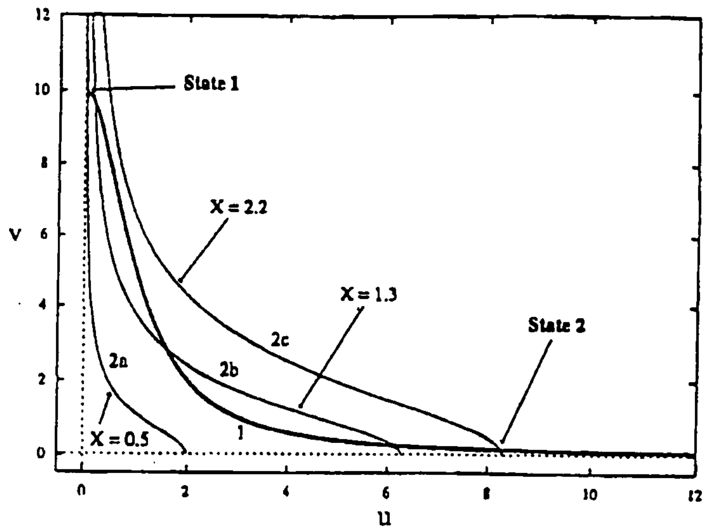


Figure 6

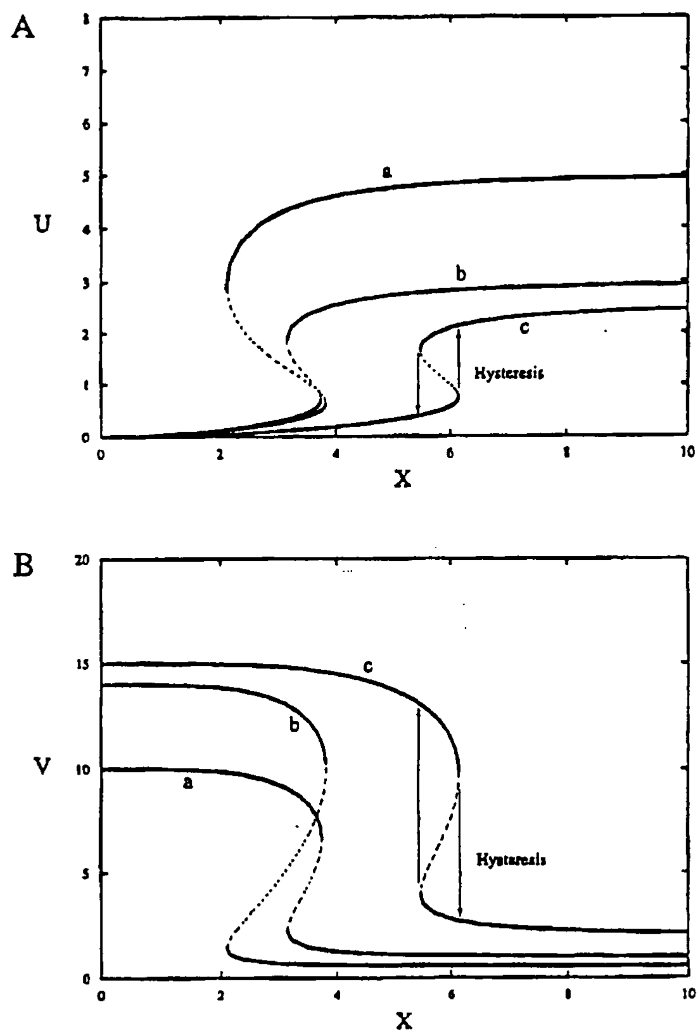


Figure 7

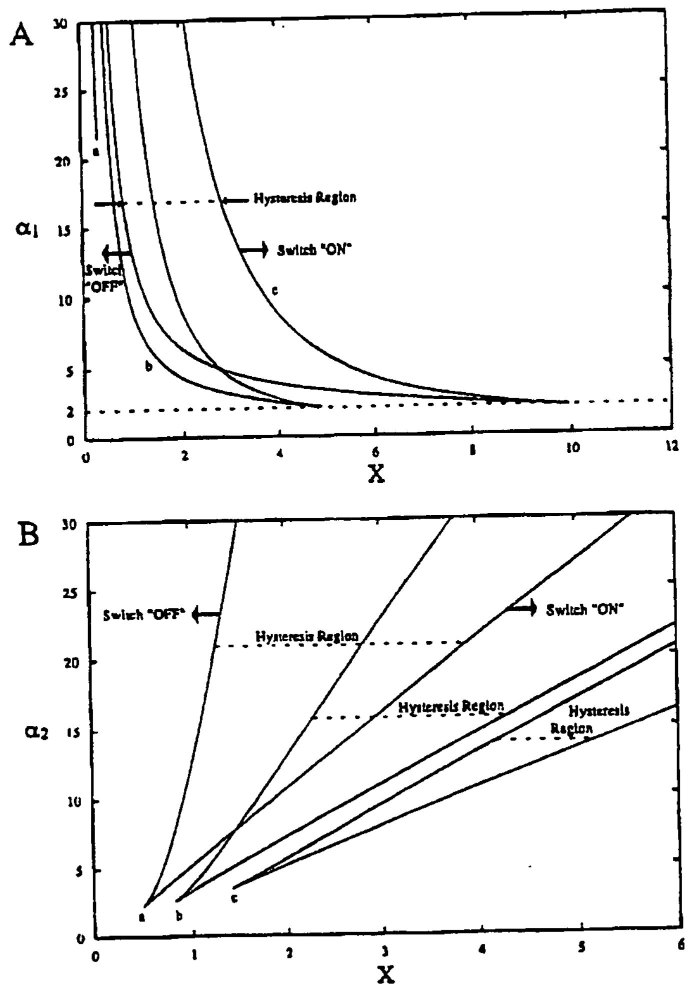

A
Hysteresis Region
Switch "ON"
Switch "OFF"
α_1
X
B
Switch "OFF"
Switch "ON"
Hysteresis Region
Hysteresis Region
Hysteresis Region
α_2
X

Figure 8

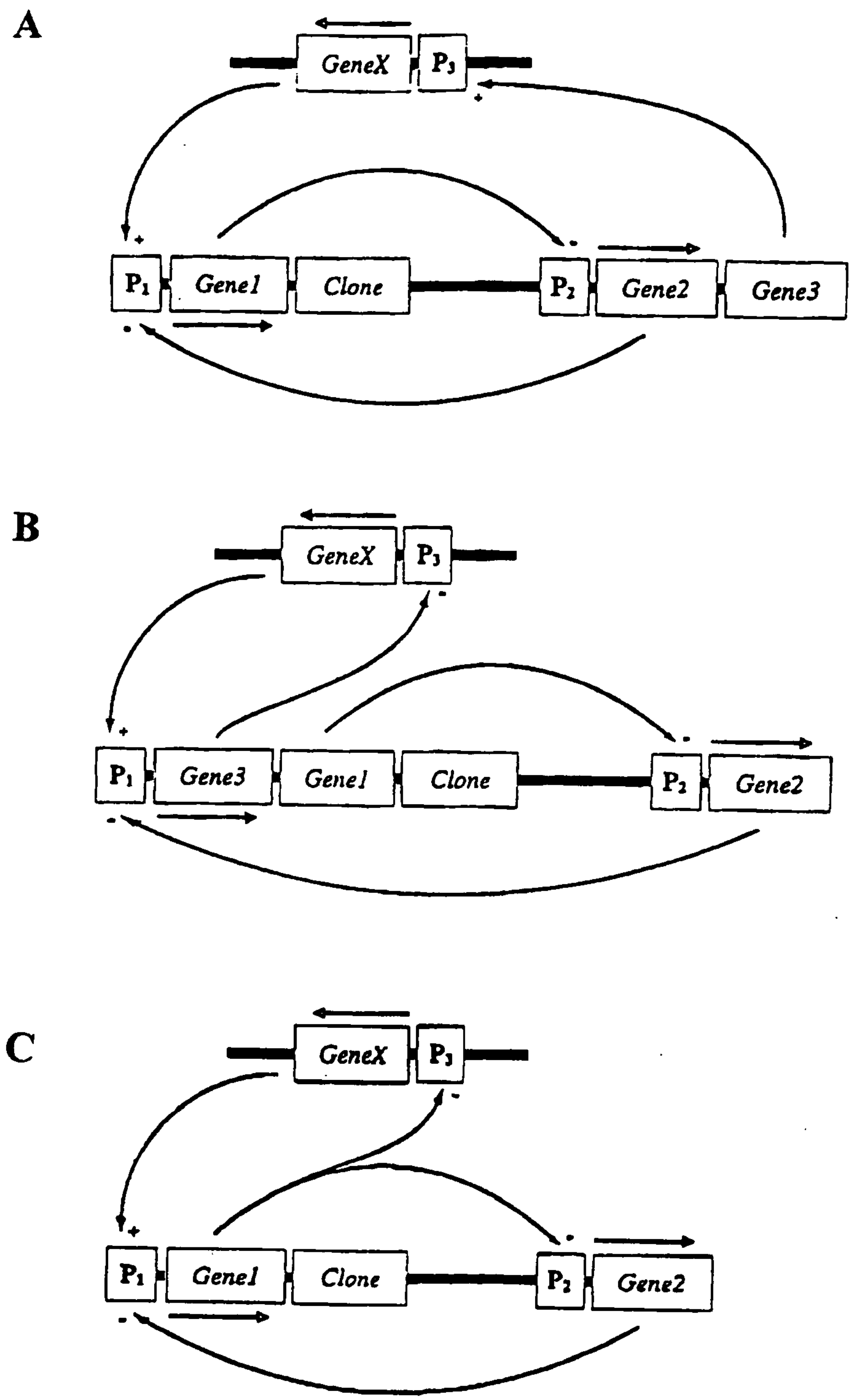

A
GeneX
P3
+
+
P1
Gene1
Clone
P2
Gene2
Gene3
B
GeneX
P3
+
P1
Gene3
Gene1
Clone
P2
Gene2
C
GeneX
P3
+
P1
Gene1
Clone
P2
Gene2

Figure 9

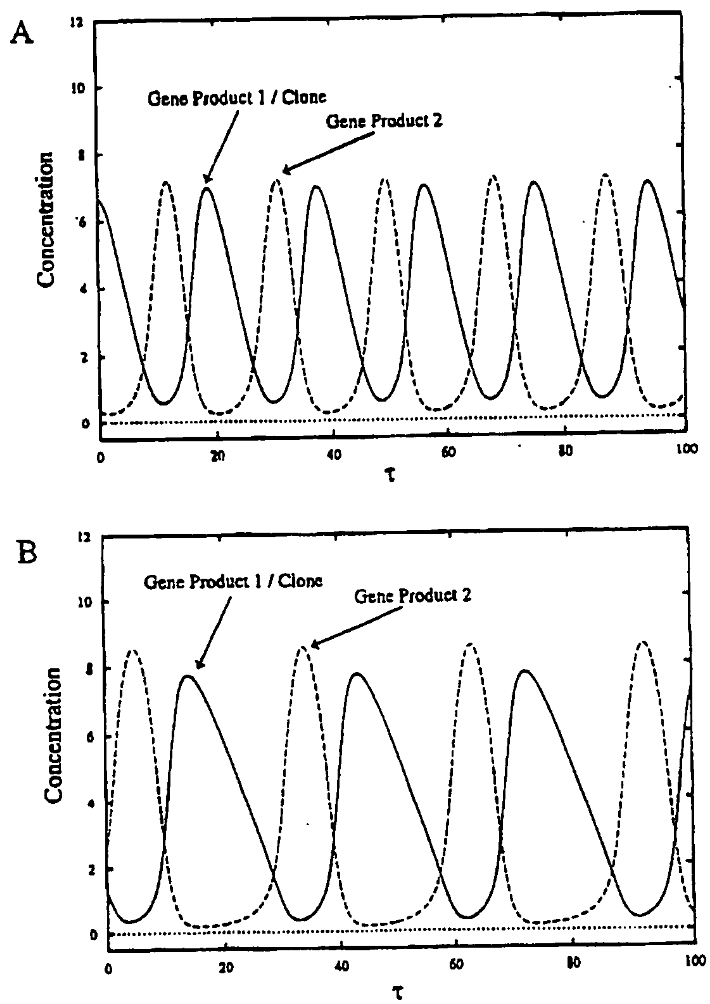

A
Gene Product 1 / Clone
Gene Product 2
Concentration
τ
B
Gene Product 1 / Clone
Gene Product 2
Concentration
τ

Figure 10

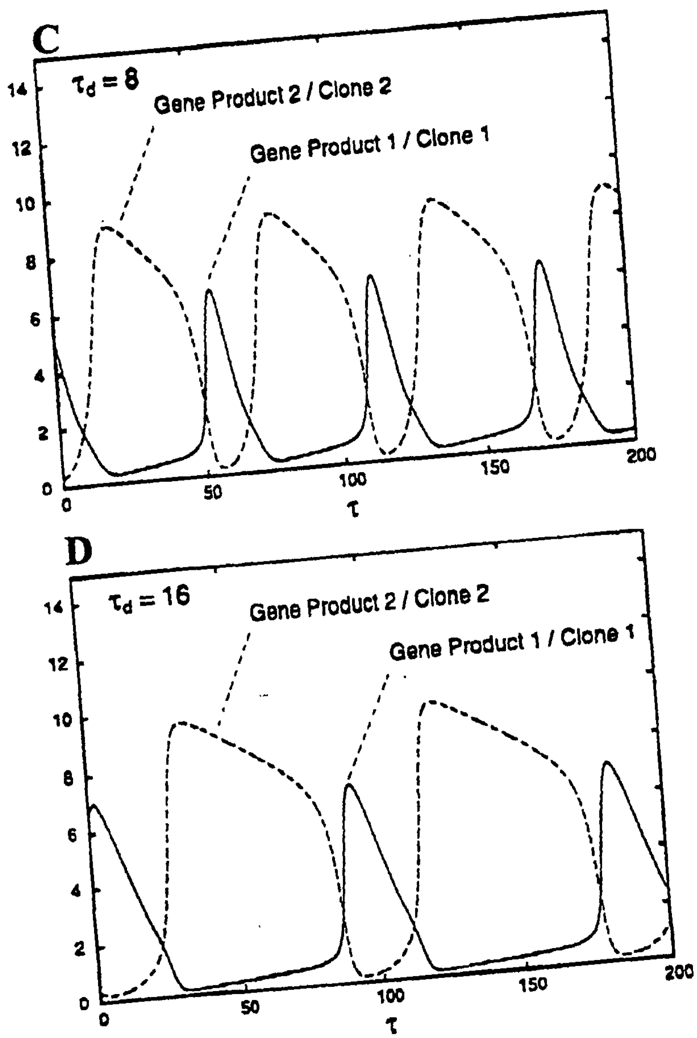

C
$\tau_d = 8$
Gene Product 2 / Clone 2
Gene Product 1 / Clone 1
0
2
4
6
8
10
12
14
0
50
100
150
200
τ
D
$\tau_d = 16$
Gene Product 2 / Clone 2
Gene Product 1 / Clone 1
0
2
4
6
8
10
12
14
0
50
100
150
200
τ

Figure 10

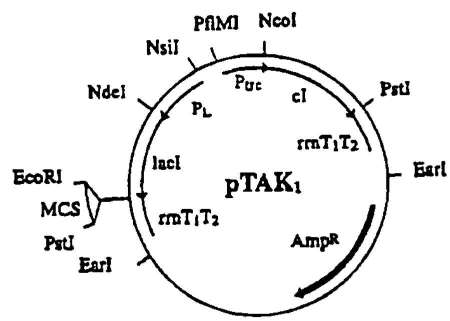

PflMI
NcoI
NsiI
NdeI
Ptrc
cI
PstI
PL
rrnT1T2
EarI
lacI
pTAK1
EcoRI
MCS
rrnT1T2
PstI
EarI
AmpR

Figure 11

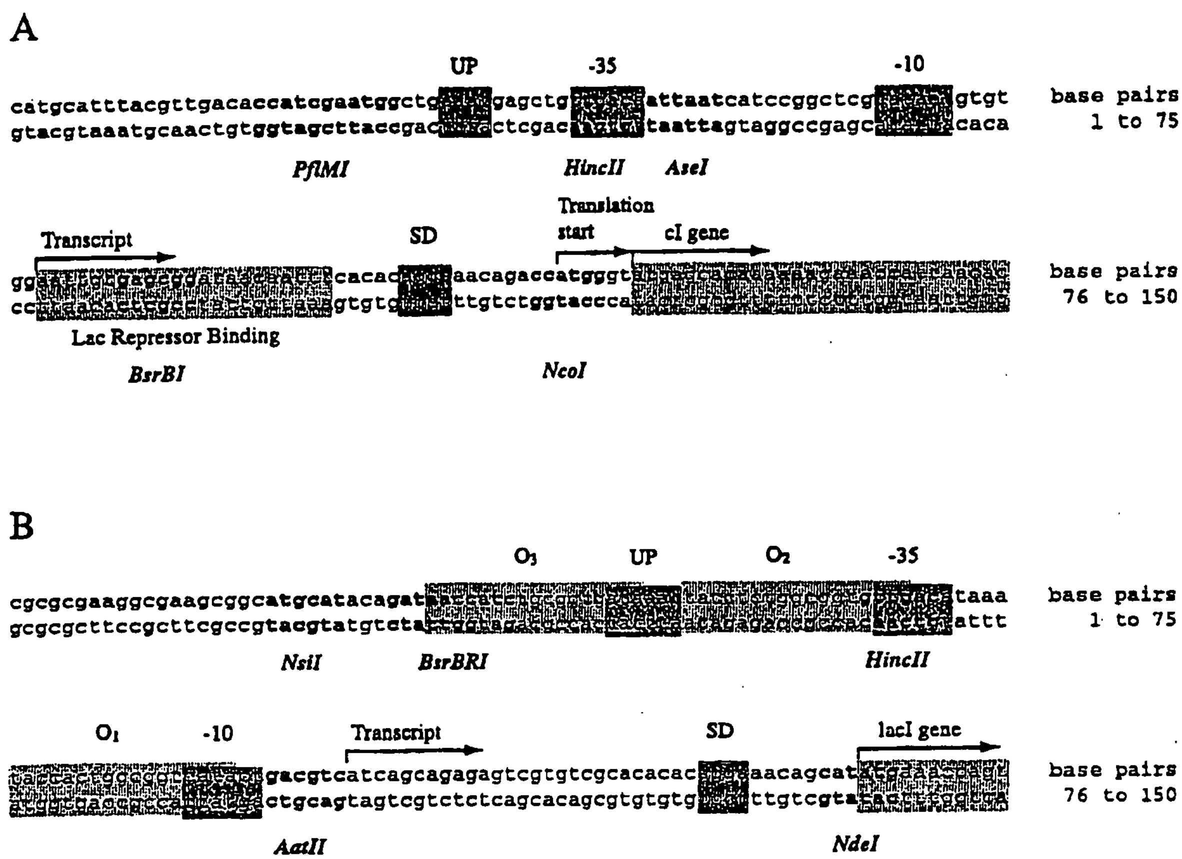

A
UP
-35
-10
base pairs 1 to 75
PflMI
HincII
AseI
Translation start
Transcript
SD
cI gene
base pairs 76 to 150
Lac Repressor Binding
BsrBI
NcoI
B
O3
UP
O2
-35
base pairs 1 to 75
NsiI
BsrBRI
HincII
O1
-10
Transcript
SD
lacI gene
base pairs 76 to 150
AatII
NdeI

Figure 12

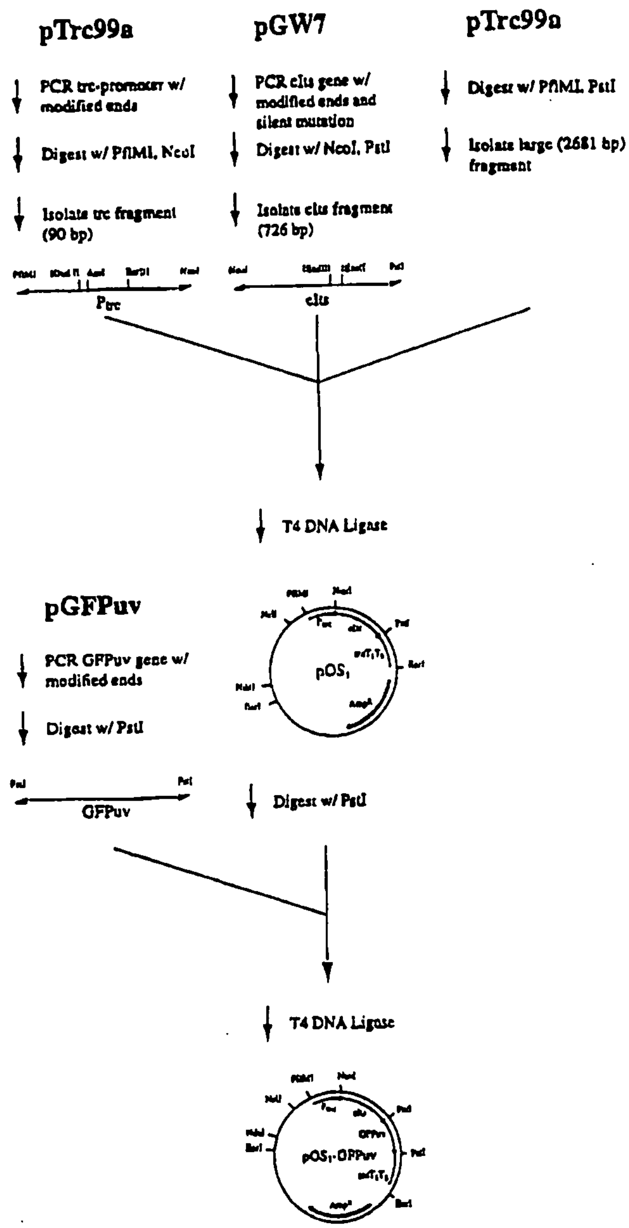

pTrc99a
PCR trc-promoter w/ modified ends
Digest w/ PflMI, NcoI
Isolate trc fragment (90 bp)
Ptrc
pGW7
PCR clts gene w/ modified ends and silent mutation
Digest w/ NcoI, PstI
Isolate clts fragment (726 bp)
clts
pTrc99a
Digest w/ PflMI, PstI
Isolate large (2681 bp) fragment
T4 DNA Ligase
pGFPuv
PCR GFPuv gene w/ modified ends
Digest w/ PstI
GFPuv
pOS1
Digest w/ PstI
T4 DNA Ligase
pOS1-GFPuv

Figure 13

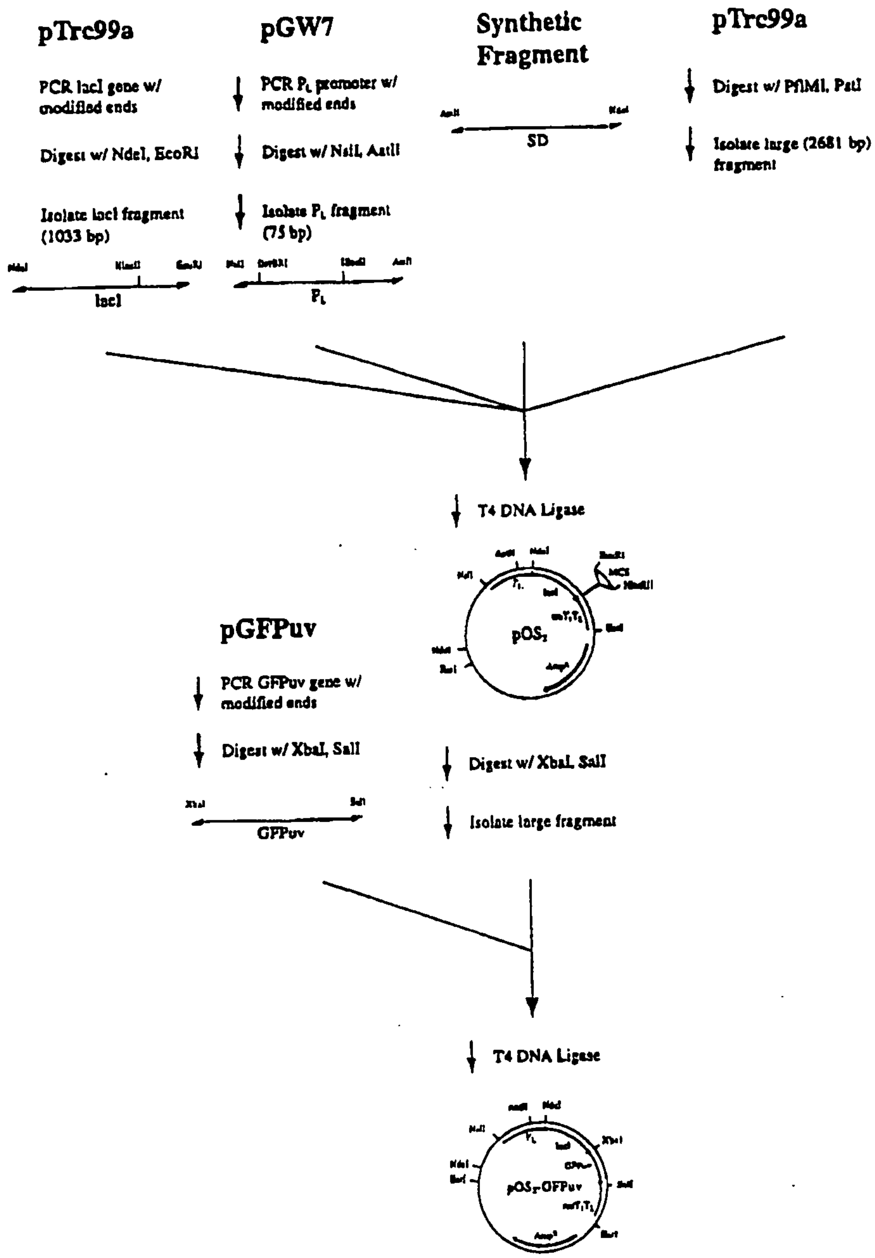

pTrc99a
PCR lacI gene w/ modified ends
Digest w/ NdeI, EcoRI
Isolate lacI fragment (1033 bp)
lacI
pGW7
PCR P_L promoter w/ modified ends
Digest w/ NsiI, AatII
Isolate P_L fragment (75 bp)
P_L
Synthetic Fragment
SD
pTrc99a
Digest w/ PflMI, PstI
Isolate large (2681 bp) fragment
T4 DNA Ligase
pOS
pGFPuv
PCR GFPuv gene w/ modified ends
Digest w/ XbaI, SalI
XbaI
SalI
GFPuv
Digest w/ XbaI, SalI
Isolate large fragment
T4 DNA Ligase
pOS-GFPuv

Figure 14

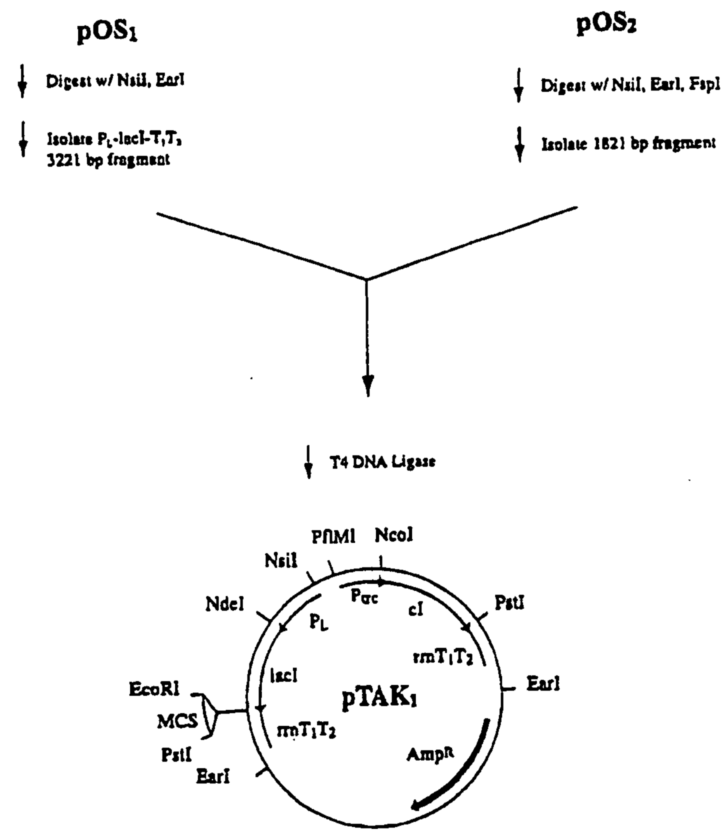

pOS1
Digest w/ NsiI, EarI
Isolate PL-lacI-T1T2 3221 bp fragment
pOS2
Digest w/ NsiI, EarI, FspI
Isolate 1821 bp fragment
T4 DNA Ligase
PflMI
NcoI
NsiI
NdeI
Ptrc
cI
PstI
PL
rrnT1T2
EarI
lacI
pTAK1
EcoRI
MCS
PstI
EarI
rrnT1T2
AmpR

Figure 15

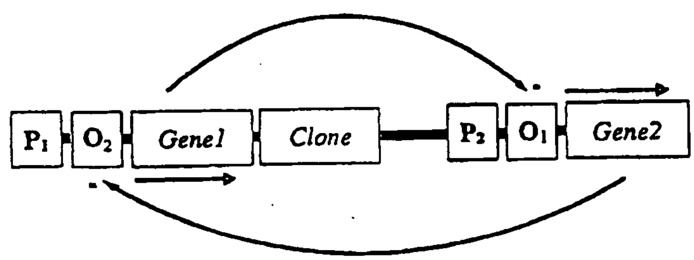


Figure 16

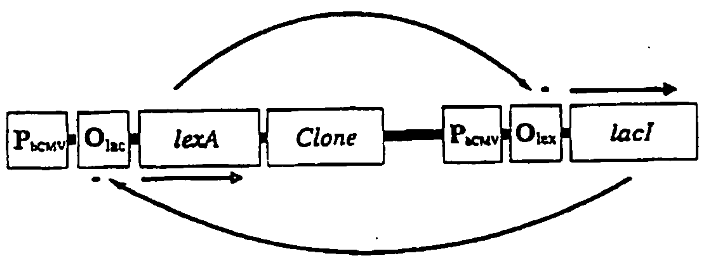


Figure 17

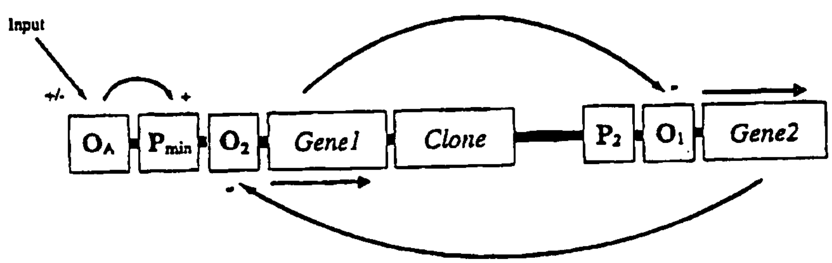


Figure 18

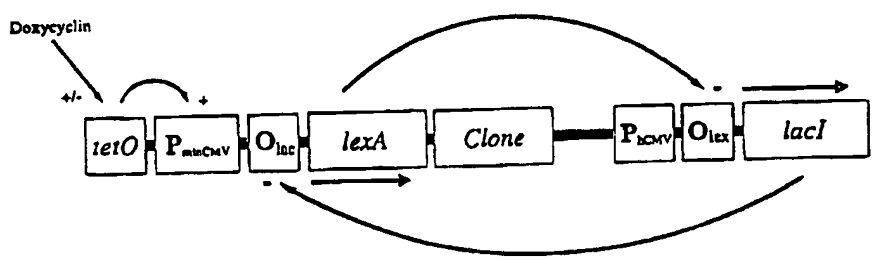


Figure 19

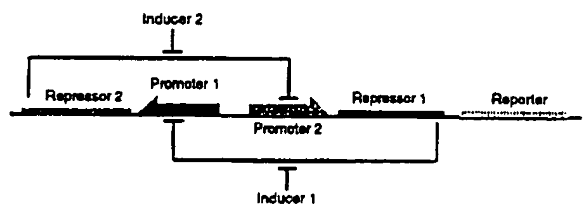

Inducer 2
Repressor 2
Promoter 1
Repressor 1
Reporter
Promoter 2
Inducer 1

Figure 20

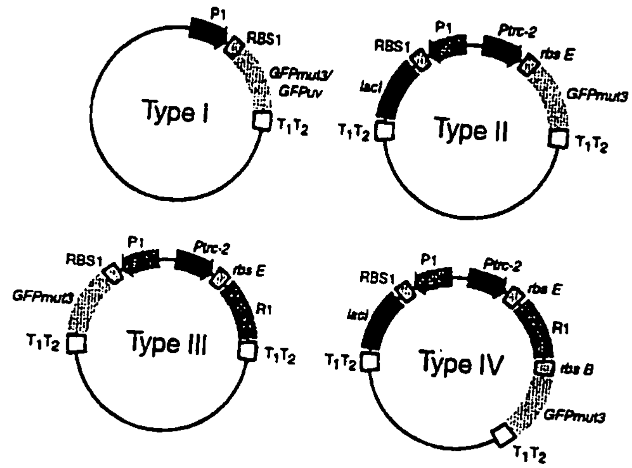

P1
RBS1
GFPmut3/
GFPuv
Type I
T_1T_2
RBS1
P1
Ptrc-2
rbs E
lacI
GFPmut3
T_1T_2
Type II
T_1T_2
RBS1
P1
Ptrc-2
rbs E
GFPmut3
R1
T_1T_2
Type III
T_1T_2
RBS1
P1
Ptrc-2
rbs E
lacI
R1
T_1T_2
Type IV
rbs B
GFPmut3
T_1T_2

Figure 21

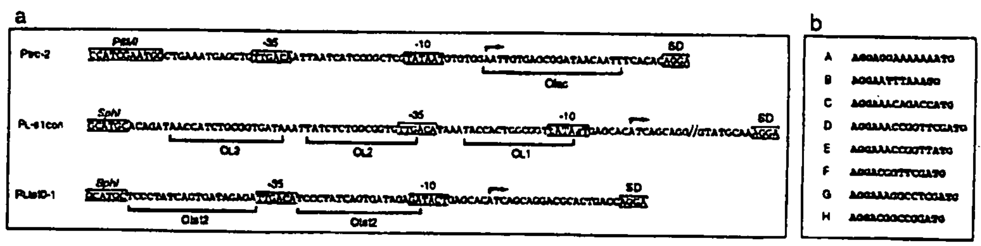


Figure 22

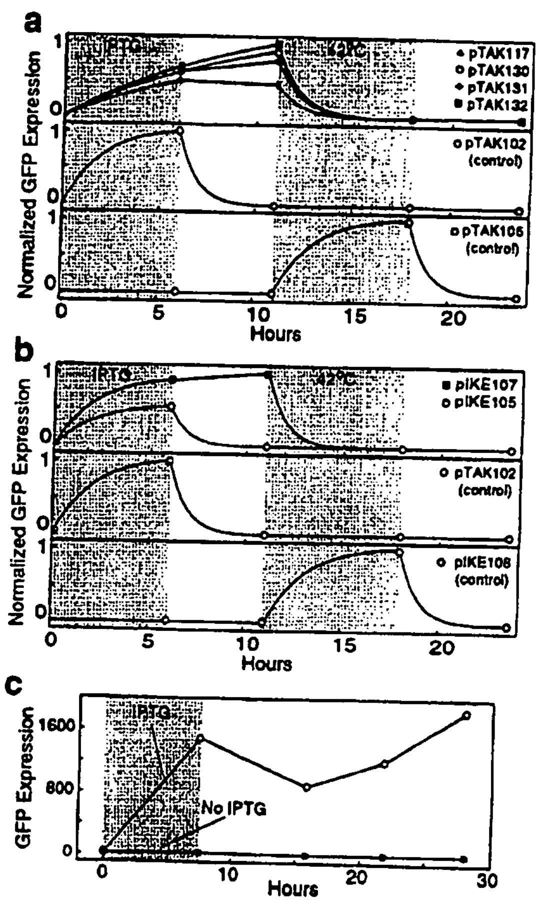

a
IPTG
42°C
pTAK117
pTAK130
pTAK131
pTAK132
pTAK102 (control)
pTAK106 (control)
Normalized GFP Expression
Hours
b
IPTG
42°C
pIKE107
pIKE105
pTAK102 (control)
pIKE108 (control)
Normalized GFP Expression
Hours
c
IPTG
No IPTG
GFP Expression
1600
800
0
Hours

Figure 23

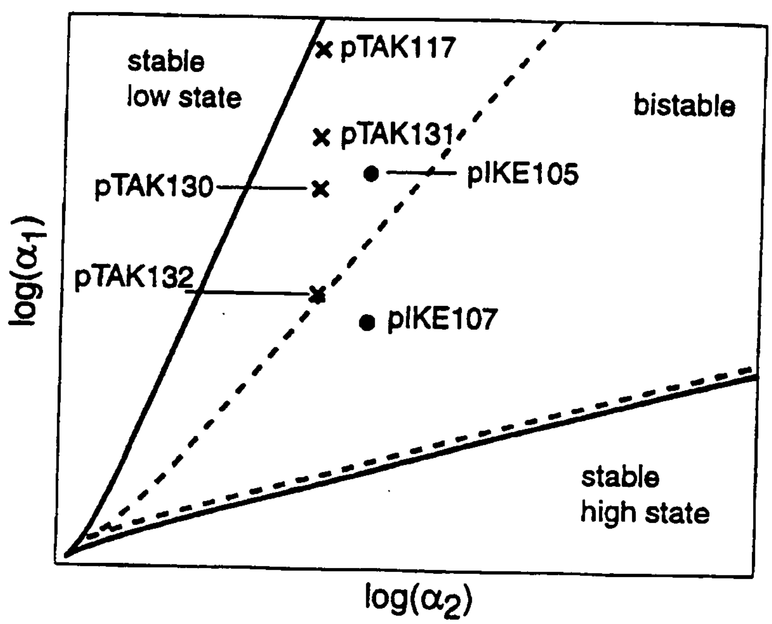

stable
low state
pTAK117
bistable
pTAK131
pTAK130
pIKE105
pTAK132
pIKE107
stable
high state
log(α1)
log(α2)

Figure 24

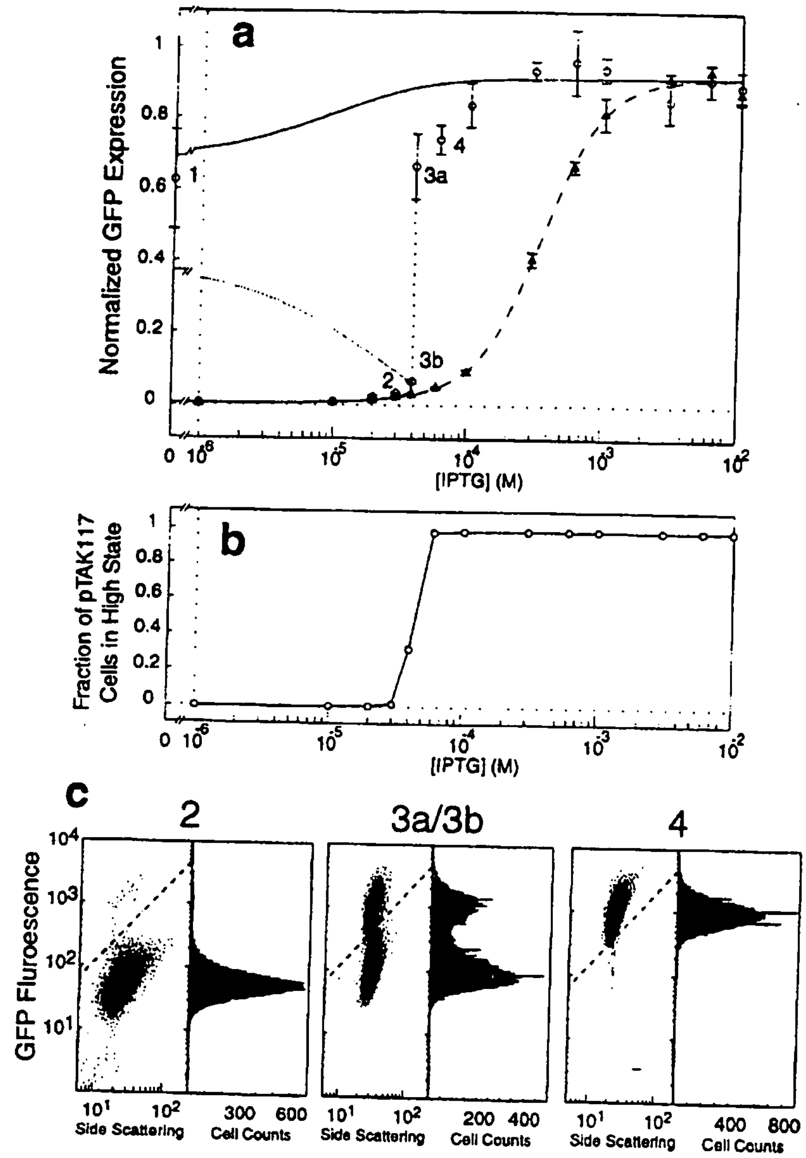

a
Normalized GFP Expression
1
0.8
0.6
0.4
0.2
0
1
3a
4
3b
2
0 10⁻⁶ 10⁻⁵ 10⁻⁴ 10⁻³ 10⁻²
[IPTG] (M)
b
Fraction of pTAK117 Cells in High State
1
0.8
0.6
0.4
0.2
0
0 10⁻⁶ 10⁻⁵ 10⁻⁴ 10⁻³ 10⁻²
[IPTG] (M)
c
2
3a/3b
4
GFP Fluroescence
10⁴
10³
10²
10¹
10¹ 10² Side Scattering
300 600 Cell Counts
10¹ 10² Side Scattering
200 400 Cell Counts
10¹ 10² Side Scattering
400 800 Cell Counts

Figure 25

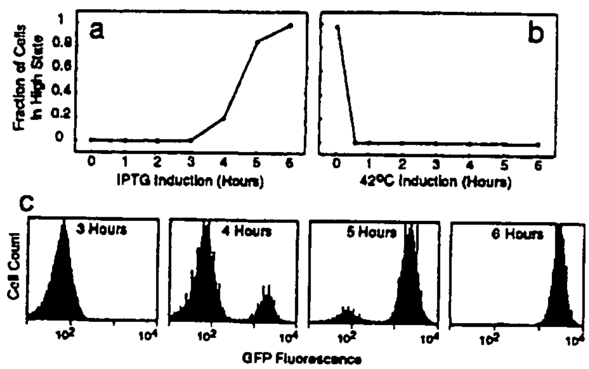
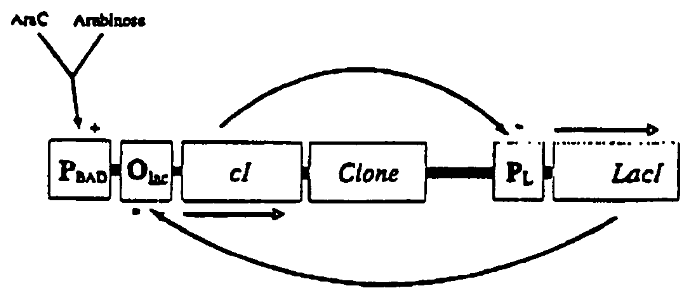

a
b
c
Fraction of Cells In High State
IPTG Induction (Hours)
42°C Induction (Hours)
3 Hours
4 Hours
5 Hours
6 Hours
Cell Count
GFP Fluorescence

Figure 26

A



B

I_1/I_2

```
gcgtcacactttgctatgccatagcatttttatccataagattagcggatcctacctgacgctttttatcgca
cgcagtgtgaaacgatacggtatcgtaaaaataggtattctaatcgcctaggatggactgcgaaaaatagcgt
```

P_{BAD}

O_{lac} SD

```
actctctactgtttctccatagatctaatgtgtggaattgtgagcggataacaatttcacacaggaaaccggt
tgagagatgacaaagaggtatctagattacacaccttaacactcgcctattgttaaagtgtgtcctttggcca
```

From P_{trc}

Figure 27